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Sir:

Transmitted herewith for filing is the patent application of:

Inventor: William Soo Hoo

For: MEMBRANE-BOUND CYTOKINE COMPOSITIONS AND METHODS OF
MODULATING AN IMMUNE RESPONSE USING SAME

Enclosed are:

☒ 4 sheets of drawing(s).☐ An assignment of the invention to _____☐ A certified copy of a _____ application.☐ A combination declaration and power of attorney.☐ A verified statement to establish small entity status under 37 C.F.R. 1.9 and 37 C.F.R. 1.27.☒ Request for Filing Application Under 37 CFR 53(a), (b) & (d).

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Andrea L. Gashler

(TYPED OR PRINTED NAME OR PERSON MAILING PAPER OR FEE)

Andrea L. Gashler
(SIGNATURE OF PERSON MAILING PAPER OR FEE)

Respectfully submitted,

Robert T. Ramos

Robert T. Ramos
Registration No. 37,915
CAMPBELL & FLORES LLP
4370 La Jolla Village Drive
Suite 700
San Diego, California 92122
619-535-9001

A P P L I C A T I O N

for

UNITED STATES LETTERS PATENT

on

MEMBRANE-BOUND CYTOKINE COMPOSITIONS AND
METHODS OF MODULATING AN IMMUNE RESPONSE USING SAME

by

William Soo Hoo

Sheets of Drawings: 4
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ANDREA L. GASHLER
(TYPED OR PRINTED NAME OR PERSON MAILING PAPER OR FEE)

Andrea L. Gashler
(SIGNATURE OF PERSON MAILING PAPER OR FEE)

Attorneys

CAMPBELL & FLORES LLP
4370 La Jolla Village Drive, Suite 700
San Diego, California 92122

MEMBRANE-BOUND CYTOKINE COMPOSITIONS AND
METHODS OF MODULATING AN IMMUNE RESPONSE USING SAME

BACKGROUND OF THE INVENTION

This invention relates generally to the fields
5 of gene therapy and cellular immunotherapy and, more
specifically, to immunomodulatory molecules such as
cytokines expressed as membrane-bound fusion proteins.

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The use of cancer cell vaccines derived from
autologous cancer cells has been explored throughout this
10 century. Unfortunately, for most patients the responses
achieved with such vaccines have been at best partial and
short-lived. Strategies to improve the efficacy of
cancer vaccines include the use of cytokines, which
are pleiotropic mediators that modulate and shape the
15 quality and intensity of the immune response. Cytokines
are occasionally autocrine or endocrine but largely
paracrine hormones produced in nature by lymphocytes and
monocytes. Several cytokines have been produced using
recombinant DNA methodology and evaluated for their
20 efficacy as anti-cancer therapeutics. Multiple
anti-tumor activities are attributed to cytokines
including (1) direct inhibition of tumor growth
(α -interferon), (2) reversal of the anergy-inducing
effects of tumor cells and expansion of new T-cell
25 effectors (interleukin-2), (3) augmentation of the
effector function of T cells to recognize MHC presented
peptide epitopes on tumor cells (granulocyte macrophage
colony stimulating factor) and (4) enhanced recruitment
of cells to inflammatory sites (interleukin-4). However,
30 many cytokines cannot be tolerated when administered at
the high systemic levels required for an effective
response, thus limiting the therapeutic value of these
agents.

Local cytokine delivery can more closely mimic the natural immune response and avoid the toxicity associated with high systemic levels of these molecules. One approach to local cytokine delivery involves the use of genetically modified tumor cells. For example, transduction of murine tumor cells with the gene for interleukin-4 (IL-4), interleukin-2 (IL-2), interferon γ (IFN- γ), tumor necrosis factor α (TNF- α), interleukin-6 (IL-6), interleukin-7 (IL-7), granulocyte colony stimulating factor (GCSF) or granulocyte macrophage colony stimulating factor (GM-CSF) can lead to rejection of genetically modified tumor cells by syngeneic hosts. Furthermore, vaccination with cytokine-secreting cells can increase systemic immunity as well, protecting vaccinated animals from challenge with non-transduced tumor cells. Unlike systemic administration, localized cytokine transgene expression is generally not associated with toxicity.

Dendritic cells form a system of highly efficient antigen-presenting cells and are central to the design of effective anti-cancer therapies. After capturing antigen in the periphery, dendritic cells migrate to lymphoid organs and present antigens to T cells. These potent antigen-presenting cells appear unique in their ability to interact with and activate naive T cells. The potent antigen-presenting capacity of dendritic cells can be due in part to their unique life cycle and high level expression of major histocompatibility complex (MHC) class I and II molecules and co-stimulatory molecules. Granulocyte macrophage colony stimulating factor (GM-CSF) molecule is a cytokine important in the maturation and function of dendritic cells: GM-CSF binds receptors on dendritic cells and stimulates these cells to mature, present antigen and prime naive T cells. Thus, the use of GM-CSF is of particular interest in immunotherapy.

Optimal stimulation of immune cells such as dendritic cells depends upon strong cytokine-receptor interactions. Enhanced stimulation of an immune response can be achieved by increasing the number of
5 cytokine-receptor pairings or by increasing the affinity of a cytokine-receptor interaction. However, increasing the natural affinity of cytokines for their receptors can be impractical, and available cytokine-secreting tumor cell vaccines are limited in their ability to produce a
10 high local concentration of cytokine. Thus, there is a need for improved cellular vaccines with increased cytokine-receptor avidity.

Cellular vaccines, including membrane-bound
15 immunostimulatory cytokines such as GM-CSF, can be used as adjuvant therapy with surgery to eliminate micro-metastases. Such cellular anti-cancer vaccines also can be administered as preventive therapy for individuals at risk for particular types of cancer, such
20 as individuals at risk for melanoma. Conversely, vaccines including immunosuppressive cytokine molecules can be used to dampen the inappropriate immune response that causes autoimmune disorders such as rheumatoid arthritis, multiple sclerosis and psoriasis.

Thus, there is a need for improved cellular
25 vaccines for protection against and treatment of cancers such as melanoma, colon or breast cancer; autoimmune diseases such as rheumatoid arthritis, psoriasis and multiple sclerosis; parasitic diseases; and infectious
30 diseases such as AIDS. The present invention satisfies this need by providing improved cellular vaccines containing membrane-bound immunomodulatory molecule such as cytokines and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides a cellular vaccine having a membrane-bound fusion protein that includes a non-antibody immunomodulatory molecule

5 operatively fused to a heterologous membrane attachment domain. Non-antibody immunomodulatory molecules useful in the invention include immunostimulatory and immunosuppressive molecules such as cytokines. In one embodiment, the invention provides a cellular vaccine

10 having a membrane-bound fusion protein that includes a non-antibody immunomodulatory molecule operatively fused to a heterologous membrane attachment domain and, additionally, a disease-associated antigen or immunogenic epitope thereof. Further provided by the invention are

15 methods of modulating an immune response against a disease-associated antigen by administering to an individual a cellular vaccine having a membrane-bound fusion protein that includes a non-antibody immunomodulatory molecule operatively fused to a

20 heterologous membrane attachment domain.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. (A) The strategy for cloning the murine GM-CSF cDNA into pHOOKTM-1 is shown. (B) The resulting pHOOKTM-1.GM-CSF expression vector is shown.

25 Figure 2 shows the amino acid sequence (SEQ ID NO:1) and nucleotide sequence (SEQ ID NO:2) of the pHOOKTM-1.GM-CSF fusion protein, which contains murine granulocyte macrophage colony stimulating factor (GM-CSF) and the human platelet derived growth factor receptor

30 (PDGFR) β chain transmembrane domain.

Figure 3 shows analysis of GM-CSF expression in pHOOK™-1.GM-CSF CT-26 cell transfectants using reverse transcriptase polymerase chain reaction (RT-PCR). Lane 1: ϕ X174 molecular weight markers. Lanes 2 through 13: CT-26 pHOOK™-1.GM-CSF transfectants A3, A5, A6, B4, B5, C2, C3, C4, C5, C6, D3 and D5. Lane 14: untransfected CT-26 cells. Lane 15: Concanavalin A stimulated Balb/c spleen cells.

Figure 4 shows exemplary double stain FACS analysis of the "C3" pHOOK™-1.GM-CSF transfected clone.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a cellular vaccine having a membrane-bound fusion protein that includes a non-antibody immunomodulatory molecule operatively fused to a heterologous membrane attachment domain. The immunomodulatory molecule can be an immunostimulatory or immunosuppressive molecule such as a cytokine. Cytokines useful in the vaccines of the invention include granulocyte macrophage colony stimulating factor (GM-CSF); granulocyte colony stimulating factor (G-CSF); interferon γ (IFN- γ); interferon α (IFN- α); tumor necrosis factor- α (TNF- α); tumor necrosis factor- β (TNF- β); interleukin-1 (IL-1); interleukin-2 (IL-2); interleukin-3 (IL-3); interleukin-4 (IL-4); interleukin-6 (IL-6); interleukin-7 (IL-7); interleukin-10 (IL-10); interleukin-12 (IL-12), lymphotactin (LTN) and dendritic cell chemokine 1 (DC-CK1).

As used herein, the term "non-antibody immunomodulatory molecule" means a molecule that modulates or regulates the production of an immune

response to an antigen. A vaccine of the invention contains one or more such non-antibody immunomodulatory molecules in membrane-bound form. Antigen recognition sequences from antibody molecules are explicitly excluded from the vaccines, methods and nucleic acid molecules of the invention. As used herein, the term "antibody" means an immunoglobulin molecule or antigen-binding fragment thereof.

As used herein, the term "immunostimulatory molecule" means an immunomodulatory molecule that promotes or enhances the production of an immune response to an antigen.

As used herein, the term "immunosuppressive molecule" means an immunomodulatory molecule that reduces or inhibits the production of an immune response to an antigen.

Cytokines are immunomodulatory molecules particularly useful in the vaccines of the invention. As used herein, the term "cytokine" refers to a member of the class of proteins that are produced by cells of the immune system and that regulate or modulate an immune response. Such regulation can occur within the humoral or the cell mediated immune response and includes modulation of the effector function of T cells, B cells, NK cells macrophages, antigen presenting cells or other immune system cells.

Cytokines typically are small proteins or glycoproteins having a molecular mass of less than about 30 kDa. Although cytokines occasionally exhibit autocrine or endocrine activity, most act in a paracrine fashion and bind specific receptors on the membrane of target cells, thereby triggering signal transduction pathways that alter gene expression. Cytokines generally

display very high affinity for their cognate receptors, with dissociation constants ranging from about 10^{-9} to 10^{-12} M. Due to this high affinity, picomolar concentrations of cytokines can mediate biological effects. Constitutive production of cytokines is usually low or absent; cytokine expression is regulated by various inducing stimuli at the level of transcription or translation. Cytokines are typically transiently expressed with secretion lasting from a few hours to a few days (Thomson, The Cytokine Handbook (Second Edition) London: Harcourt Brace & Company (1994); Callard and Gearing, The Cytokine Facts Book Academic Press, Inc. (1994); Kuby, Immunology (Third Edition) New York: W.H. Freeman and Company (1997), each of which are incorporated herein by reference). Exemplary cytokines useful in the vaccines of the invention are shown in Table 1.

As used herein, the term cytokine encompasses those cytokines secreted by lymphocytes and other cell types (designated lymphokines) as well as cytokines secreted by monocytes and macrophages and other cell types (designated monokines). The term cytokine includes the interleukins, such as IL-2, IL-4 and IL-12, which are molecules secreted by leukocytes that primarily affect the growth and differentiation of hematopoietic and immune-system cells. The term cytokine also includes hematopoietic growth factors and, in particular, colony stimulating factors such as colony stimulating factor-1, granulocyte colony stimulating factor and granulocyte macrophage colony stimulating factor. In addition, the term cytokine encompasses chemokines, which are low-molecular weight molecules that mediate the chemotaxis of various leukocytes and can regulate leukocyte integrin expression or adhesion. Exemplary chemokines include interleukin-8, dendritic cell chemokine 1 (DC-CK1) and lymphotactin, which is a

chemokine important for recruitment of $\gamma\delta$ T cells and for mucosal immunity, as well as other members of the C-C and C-X-C chemokine subfamilies (see, for example, Miller and Krangel, Crit. Rev. Immunol. 12:17-46 (1992); Schall, "The Chemokines" in Thomson, *supra*, 1994; Hedrick et al., J. Immunol. 158:1533-1540 (1997); and Boismenu et al., J. Immunol. 157:985-992 (1996), each of which are incorporated herein by reference).

The term cytokine, as used herein, encompasses cytokines produced by the T helper 1 (T_H1) and T helper 2 (T_H2) subsets. Cytokines of the T_H1 subset are produced by T_H1 cells and include IL-2, IL-12, IFN- α and TNF- β . Cytokines of the T_H1 subset are responsible for classical cell-mediated functions such as activation of cytotoxic T lymphocytes and macrophages and delayed-type hypersensitivity. Cytokines of the T_H1 subset are particularly useful in stimulating an immune response to tumor cells, infected cells and intracellular pathogens.

Cytokines of the T_H2 subset are produced by T_H2 cells and include the cytokines IL-4, IL-5, IL-6 and IL-10. Cytokines of the T_H2 subset function effectively as helpers for B-cell activation and are particularly useful in stimulating an immune response against free living bacteria and helminthic parasites. Cytokines of the T_H2 subset also can mediate allergic reactions.

Active fragments of immunomodulatory molecules, for example active fragments of cytokines, also are useful in the vaccines of the invention. Such active fragments are polypeptide fragments having substantially the same amino acid sequence as a portion of the indicated immunomodulatory molecule, provided that the fragment retains at least one biological activity of the immunomodulatory molecule. Active cytokine fragments are known in the art and include, for example, a nine-amino

acid peptide from IL-1 β (VQGEESNDK; SEQ ID NO:3), which retains the immunostimulatory activity of the full-length IL-1 β cytokine (Hakim et al., J. Immunol. 157:5503-5511 (1996), which is incorporated herein by reference). In addition, a variety of well known *in vitro* and *in vivo* assays for cytokine activity, such as the bone marrow proliferation assay described in Example I, are useful in testing a cytokine fragment for activity (see Thomson, *supra*, 1994).

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15

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Table 1	
EXEMPLARY CYTOKINES	
Cytokine	Reference
Interleukin-1 (IL-1 α , IL-1 β)	Dinareello, <u>Adv. Immunol.</u> 44:153-205 (1989)
Interleukin-2 (IL-2)	Devos et al., <u>Nucl. Acids Res.</u> 11:4307-4323 (1983)
Interleukin-3 (IL-3)	Yang et al., <u>Cell</u> 47:3-10 (1986)
Interleukin-4 (IL-4)	Yakota et al., <u>Proc. Natl. Acad. Sci., USA</u> 83:5894-5898 (1986)
Interleukin-5 (IL-5)	Harada et al., <u>J. Immunol.</u> 134:3944-3951 (1985)
Interleukin-6 (IL-6)	Hirano et al., <u>Nature</u> 324:73-76 (1986)
Interleukin-7 (IL-7)	Goodwin et al., <u>Proc. Natl. Acad. Sci., USA</u> 86:302-306 (1989)

TABLE 1 CONTINUED	
EXEMPLARY CYTOKINES	
Cytokine	Reference
5 Interleukin-8 (IL-8)	Schmid and Weissmann, <u>J. Immunol.</u> 139:250-256 (1987)
Interleukin-9 (IL-9)	Yang et al., <u>Blood</u> 74:1880-11-884 (1989)
Interleukin-10 (IL-10)	Vieira et al., <u>Proc. Natl. Acad. Sci., USA</u> 88:1172-1176 (1991)
Interleukin-11 (IL-11)	Paul et al., <u>Proc. Natl. Acad. Sci., USA</u> 87:7512-7516 (1990)
Interleukin-12 (IL-12)	Wolf et al., <u>J. Immunol.</u> 146:3074-3081 (1991)
10 Interleukin-13 (IL-13)	Cherwinski et al., <u>J. Exp. Med.</u> 166:1229-1244 (1987) Brown et al., <u>J. Immunol.</u> 142:679-687 (1989)
Interleukin-14 (IL-14)	Ambrus et al., <u>Proc. Natl. Acad. Sci., USA</u> 90:6330-6334 (1993)
Interleukin-15 (IL-15)	Grabstein et al., <u>Science</u> 264:965-968 (1994)
Interleukin-16 (IL-16)	Baier et al., <u>Proc. Natl. Acad. Sci., USA</u> 94:5273-5279 (1997)
Interferon- α (IFN- α)	Pestka et al., <u>Annu. Rev. Biochem.</u> 56:727-777 (1987)
15 Interferon- β (IFN- β)	Pestka et al., <i>supra</i> , 1987

TABLE 1 CONTINUED		
EXEMPLARY CYTOKINES		
	Cytokine	Reference
5	Interferon- γ (IFN- γ)	Vilcek et al., <u>Lymphokines</u> 11:1-32 (1985)
	Leukemia-inhibitory factor (LIF)	Gearing et al., <u>Annals NY Acad. Sci.</u> 628918 (1991)
	Oncostatin M (OSM)	Malik et al., <u>Mol. Cell. Biol.</u> 9:2847-2853 (1989)
10	Transforming growth factor β (TGF- β)	Sporn and Roberts (Eds), <u>Handbook of Experimental Phar.</u> Springer-Verlag Vol 65: 419-472
	Tumor necrosis factor- α (TNF- α)	Wang et al., <u>Science</u> 228:149-154 (1985)
	Tumor necrosis factor- β (TNF- β)	Gray et al., <u>Nature</u> 312:721-724 (1984)
15	Dendritic cell chemokine 1 (DC-CK1)	Adema et al., <u>Nature</u> 387:713-717 (1997)
20	Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)	Lee et al., <u>Proc. Natl. Acad. Sci., USA</u> 82:4360-4364 (1985)
	Colony Stimulating Factor 1 (CSF-1)	Kawasaki et al., <u>Science</u> 230:291-296 (1985)
25	Granulocyte Colony Stimulating Factor (GCSF)	Negata et al., <u>Nature</u> 319:415-418 (1986)
	Macrophage chemotactic and activating factor (MCAF)	Furutani et al., <u>Biochem. Biophys. Res. Comm.</u> 159:249-255 (1989)

TABLE 1 CONTINUED	
EXEMPLARY CYTOKINES	
Cytokine	Reference
5 Macrophage inflammatory protein-1 (MIP-1)	Zipfel et al., <u>J. Immunol.</u> 142:1582-1590 (1989) Blum et al., <u>DNA Cell. Biol.</u> 9:589-602 (1990)
Macrophage inflammatory protein-1 (MIP-1)	Lipes et al., <u>Proc. Natl. Acad. Sci., USA</u> 85:9704-9708 (1988); Brown et al., <u>J. Immunol.</u> 142:679-687 (1989)
RANTES	Schall et al., <u>J. Immunol.</u> 141:1018-1025 (1988)
10 Neutrophil-activating protein (NAP-2)	Walz et al., <u>J. Exp. Med.</u> 170:1745-1750 (1989)
Platelet factor 4 (PF-4)	Poncz et al., <u>Blood</u> 69:219-223 (1987)

An immunomodulatory molecule can have the
 15 sequence of a naturally occurring immunomodulatory molecule or can have an amino acid sequence with substantial amino acid sequence similarity to the sequence of a naturally occurring immunomodulatory molecule. Thus, it is understood that limited
 20 modifications to a naturally occurring sequence can be made without destroying the biological function of an immunomodulatory molecule. For example, minor modifications of GM-CSF that do not destroy polypeptide activity fall within the definition of GM-CSF. These
 25 modifications can be deliberate, as through site-directed mutagenesis, or can be accidental such as through mutation in hosts harboring an encoding nucleic acid. All such modified polypeptides are included in the definition of an immunomodulatory molecule as at least

one biological function of the immunomodulatory molecule is retained.

A cytokine antagonist also can be an immunomodulatory molecule useful in the invention. Such cytokine antagonists can be naturally occurring or non-naturally occurring and include, for example, antagonists of GM-CSF, G-CSF, IFN- γ , IFN- α , TNF- α , TNF- β , IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-12, lymphotactin and DC-CK1. Cytokine antagonists include cytokine deletion and point mutants, cytokine derived peptides, and soluble, dominant negative portions of cytokine receptors. Naturally occurring antagonists of IL-1, for example, can be used in a vaccine of the invention to inhibit the pathophysiological activities of IL-1. Such IL-1 antagonists include IL-1Ra, which is a polypeptide that binds to IL-1 receptor I with an affinity roughly equivalent to that of IL-1 α or IL-1 β but that does not activate the receptor (Fischer et al., Am. J. Physiol. 261:R442-R449 (1991); Dinarello and Thomson, Immunol. Today 12:404-410 (1991), each of which are incorporated herein by reference). IL-1 antagonists also include IL-1 β derived peptides and IL-1 muteins (Palaszynski et al., Biochem. Biophys. Res. Commun. 147:204-209 (1987), which is incorporated herein by reference). Cytokine antagonists useful in the invention also include, for example, antagonists of TNF- α (Ashkenazi et al., Proc. Natl. Acad. Sci., USA 88:10535-10539 (1991); Mire-Sluis, Trends in Biotech. 11:74-77 (1993), each of which are incorporated herein by reference).

Heat shock proteins (HSPs) also are immunomodulatory molecules useful in the vaccines and methods of the invention. Heat shock proteins, which are induced by stress-causing conditions such as heat shock or glucose deprivation, can produce a generalized

anti-inflammatory response, thereby aiding in elimination of, for example, tumor cells or infected cells.

Heat shock proteins are distinguished by their molecular mass and grouped in families and include HSP110, HSP90,

5 HSP70, HSP60, HSP25, HSP20 and HSP8.5. Several heat shock proteins, including HSP60, HSP70 and HSP90, are expressed on the cell surface of mycobacteria-infected, HIV-infected cells or tumor cells (Multhoff et al, Int. J. Cancer 61:1-8 (1995), which is incorporated herein by
10 reference). The mycobacterial heat shock protein HSP65 (Silva et al., Infect. Immun. 64:2400-2407 (1996), which is incorporated herein by reference) is an example of an immunomodulatory molecule useful in the vaccines of the invention.

15 The term "membrane attachment domain," as used herein, refers to a domain that spans the width of a cell membrane, or any part thereof, and that functions to attach a polypeptide to a cell membrane. Membrane attachment domains useful in the vaccines of the
20 invention are those domains that function to attach a polypeptide to a cell surface membrane, such as the plasma membrane of an eukaryotic cell or the outer membrane of a prokaryotic cell. One skilled in the art understands that an appropriate membrane attachment
25 domain is selected based on the type of cell in which the membrane-bound fusion protein is to be expressed.

A variety of naturally occurring and synthetic membrane attachment domains derived from eukaryotic and prokaryotic cell surface proteins are useful in the
30 vaccines of the invention. For use in higher eukaryotic cells such as mammalian cells, a membrane attachment domain can be, for example, the membrane-spanning region of an integral membrane protein such as a cell surface receptor or cell adhesion molecule. Membrane attachment
35 domains useful in the invention can be derived, for

example, from cell surface receptors including growth factor receptors such as platelet derived growth factor receptor, epidermal growth factor receptor or fibroblast growth factor receptor; hormone receptors; cytokine receptors and T cell receptor. Membrane attachment domains useful in the invention also can be derived from cell adhesion molecules such as cadherins, integrins, selectins and members of the immunoglobulin superfamily; as well as other integral membrane proteins such as CD antigens. The amino acid sequences of exemplary membrane attachment domains are provided in Table 2 (see, also Pigott and Power, The Adhesion Molecule Facts Book San Diego: Academic Press, Inc. (1993) and Barclay et al., The Leukocyte Antigen Facts Book San Diego: Academic Press, Inc. (1993), each of which is incorporated herein by reference). If desired, the fusion protein can include the cytosolic domain, or portion thereof, of the heterologous protein from which the membrane attachment domain is derived.

Type I membrane attachment domains are transmembrane sequences of about 25 hydrophobic amino acid residues usually followed by a cluster of basic amino acids. Amino acids that are usually excluded from such membrane attachment domains include Asn, Asp, Glu, Gln, His, Lys and Arg, although where the domains form a multimeric complex in the membrane, there can be charged residues present. The orientation of a type I membrane attachment domain is such that the amino-terminal portion is extracellular. Such type I membrane attachment domains can be derived, for example, from CD2, CD40 or the IL-4 receptor.

Type II membrane attachment domains are transmembrane domains useful in the vaccines of the invention. The orientation of a type II membrane attachment domain is such that the carboxy-terminal

portion is extracellular. Examples of type II membrane attachment domains include the transmembrane domain of CD72.

A membrane attachment domain of the invention also can be a phosphatidylinositol-glycan (PI-G) anchor, which is attached to the carboxy-terminal residue of a protein. A PI-G anchor can be derived, for example, from human placental alkaline phosphatase (HPAP), and can function to anchor a fusion protein to the cell surface (see, for example, Whitehorn et al., Biotechnology 13:1215-1219 (1995), which is incorporated herein by reference). PI-G-anchored molecules have a signal sequence at their carboxy-terminus that is cleaved off and replaced by the PI-G anchor. The residues at the PI-G attachment site and immediately following are typically small amino acids such as Ala, Asn, Asp, Gly, Cys or Ser. After the attachment residue, there is a hydrophobic sequence of about 10 to 20 residues starting 7-10 residues after the attachment point. Such hydrophobic PI-G-signal sequences generally lack the basic charged residues found in type I membrane attachment domains.

Type III membrane attachment domains, or segments thereof, also can be useful in the vaccines of the invention. Such type III membrane attachment domains are derived from eukaryotic cell surface molecules that cross the lipid bilayer numerous times. A membrane attachment domain useful in the invention can be, for example, one or more transmembrane domains derived from MDR1, a G-protein linked receptor or a protein of the rhodopsin superfamily.

Table 2		
Exemplary Membrane Attachment Domains		
Source	SIN:	Sequence of membrane attachment domain
P-Cadherin	4	FILPILGAVLALLLLLTLLALLLV
CD2	5	IYLIIGICGGGSLLMVFVALLVFIYIT
CD40	6	ALVVIPIIFGILFAILLVLFVI
Contactin	7	ISGATAGVPTLLLGLVLPAP
IL-4 receptor	8	LLLGVSVSCIVILAVCLLCYVSIT
Mannose receptor	9	VAGVVIIVILLILTGAGLAAYFFY
M-CSF receptor	10	FLFTPVVVACMSIMALLLLLLLLLLL
PDGFR β chain	11	VVVISAILALVVLTIISLIILIMLWQK KPR
PDGFR α chain	12	ELTVAAAVLVLLVIVSISLIVLVVTW
P-Selectin	13	LTYFGGAVASTIGLIMGGTLLALL
Rat Thy-1	14	VKCGGISLLVQNTSWLLLLLLLSLFLQ ATDFISL
TNFR-1	15	TVLLPLVIFFGCLLSLLFIGLM
VCAM-1	16	LLVLYFASSLIIPAIGMIYFAR

A membrane attachment domain useful in a bacterial vaccine of the invention can be derived, for example, from outer membrane protein A (OmpA). For example, a transmembrane domain containing amino acids 46 to 159 of OmpA, which encodes five of the eight membrane-spanning segments of the native protein, can be a membrane attachment domain particularly useful in the invention (Francisco et al., Proc. Natl. Acad. Sci., USA 89:2713-2717 (1992); Francisco et al., Biotechnol. 11:491-495 (1993); Francisco et al., Proc. Natl. Acad. Sci., USA 90:10444-10448 (1993); Francisco and Georgiou,

Annals New York Acad. Sci. 745:372-382 (1994), each of which are incorporated herein by reference).

The term "heterologous," as used herein in reference to a membrane attachment domain operatively
5 fused to a non-antibody immunomodulatory molecule, means a membrane attachment domain derived from a source other than the gene encoding the non-antibody immunomodulatory molecule. A heterologous membrane attachment domain can be synthetic or can be encoded by a gene distinct from
10 the gene encoding the non-antibody immunomodulatory molecule to which it is fused.

The term "operatively fused," as used herein in reference to a non-antibody immunomodulatory molecule and a heterologous membrane attachment domain, means that the
15 immunomodulatory molecule and membrane attachment domain are fused in the correct reading frame such that, under appropriate conditions, a full-length fusion protein is expressed. One skilled in the art would recognize that such a fusion protein can comprise, for example, an
20 amino-terminal immunomodulatory molecule operatively fused to a carboxyl-terminal heterologous membrane attachment domain or can comprise an amino-terminal heterologous membrane attachment domain operatively fused to a carboxyl-terminal immunomodulatory molecule.

25 The term "membrane-bound," as used herein in reference to a fusion protein of the invention, means stably attached to a cellular membrane. In a vaccine of the invention, a membrane-bound fusion protein of the invention is expressed on the surface of a cell.

30 The term "fusion protein," as used herein, means a hybrid protein including a synthetic or heterologous amino acid sequence. A fusion protein can

be produced, for example, from a hybrid gene containing operatively linking heterologous gene sequences.

The term "cell," as used herein in reference to a vaccine of the invention, means any prokaryotic or eukaryotic cell capable of having expressed on its cell surface a membrane-bound fusion protein. The term cell includes live, attenuated and killed cells and encompasses primary cells, normal cells, immortalized cells, transformed cells, tumor cells or infected cells. In the methods of the invention, a cell can be autologous, allogeneic or xenogeneic to the individual to whom the vaccine is administered. Cells useful in the vaccines of the invention include mammalian cells and, in particular, human cells of a variety of cell types. In addition, the cellular vaccines of the invention can be made from bacterial cells such as *Escherichia coli*, *Salmonella*, *Listeria monocytogenes* and *Mycobacterium bovis*.

Tumor cells to be genetically modified can be obtained, for example, by biopsy from a subject having cancer, and the tumor cells subsequently modified to contain a membrane-bound fusion protein including a non-antibody immunomodulatory molecule operatively fused to a heterologous transmembrane domain. Alternatively, donor tumor cells or cells from a tumor cell line can be genetically modified to produce a vaccine of the invention.

A variety of tumor cells, especially human tumor cells such as melanoma cells, colon tumor cells, breast tumor cells, prostate tumor cells, glioblastoma cells, renal carcinoma cells, neuroblastoma cells, lung cancer cells, bladder carcinoma cells, plasmacytoma or lymphoma cells, for example, can be genetically engineered to express a membrane-bound fusion protein

including a non-antibody immunomodulatory molecule
operatively fused to a heterologous membrane attachment
domain. In a vaccine to protect against or treat
melanoma, human melanoma cell lines such as the M12, M24,
5 M101 and SK-MEL cell lines can be useful in preparing a
vaccine of the invention (Chi et al., Amer. J. Pathol.
150:2143-2152 (1997), which is incorporated herein by
reference).

10 The vaccines of the invention also can be used
to protect against or treat colon cancer. Colon tumor
cells can be obtained from culturing resected tumors or
from established human colon tumor cells lines such as
HCT 116, Colo205, SW403 or SW620. Such cells are
available to one skilled in the art, for example, from
15 the American Type Culture Collection (ATCC; Rockville,
MD).

20 The vaccines of the invention also can be used
to protect against or treat breast cancer. Primary
breast tumor cells cultured from surgically resected
tumors or human breast tumor cell lines such as the BT-20
line also can be useful preparing vaccines for protection
against and treatment of breast cancer.

25 A vaccine of the invention also can be used to
protect against or treat prostate cancer, which is the
second most frequent tumor of males in the United States.
Prostate cells for used in such vaccines can be primary
prostate cells obtained from surgically resected tumors
or can be a prostate tumor cell line such as the LNCaP
line (see Horoszewicz et al., Cancer Res. 43:1809 (1983),
30 which is incorporated herein by reference).

For protection against or treatment of brain
tumors, one can prepare a vaccine of the invention using
primary human glioma cells or cells from established

human glioblastoma or astrocytoma lines. Primary cultures of glioma cells can be established from surgically resected tumor tissue as described in Wakimoto et al., Japan. J. Cancer Res. 88:296-305 (1997), which is
5 incorporated herein by reference. Human glioblastoma cell lines, such as U-87 MG or U-118 MG, or human astrocytoma lines, such as CCF-STTG1 or SW1088 (Chi et al., *supra*, 1997), can be obtained from ATCC. Any of
10 such cells can be used to produce a vaccine that contains an immunomodulatory molecule such as GM-CSF, IL-2, IL-4, IL-6, IL-7, TNF- α or IFN- γ for protection against or treatment of human brain tumors.

It is recognized that the tumor cells to be administered can be viable. However, one skilled in the
15 art understands that administration of a viable tumor cell vaccine to a subject requires that the tumor cells be inactivated so they do not grow in the subject. Inactivation can be accomplished by any of various methods, including, for example, by irradiation, which is
20 administered to the cells at a dose that inhibits the ability of the cells to replicate but does not initially kill the tumor cells (see Example II). Such viable tumor cells can express a membrane-bound fusion protein but cannot proliferate to form new tumors.

25 Non-transformed cells including fibroblasts, myoblasts, leukocytes, hepatocytes, endothelial cells and dendritic cells, and especially non-transformed human cells, also are useful in the vaccines of the invention.
30 In particular, where a disease-associated antigen or immunogenic epitope has been isolated, a fibroblast-based vaccine of the invention can be engineered to include the disease-associated antigen or immunogenic epitope of interest. Such disease-associated antigens and
35 immunogenic epitopes thereof, including tumor-associated antigens and autoimmune disease-associated antigens, are

described further below. Fibroblasts useful in the invention include autologous fibroblasts obtained from the individual to be vaccinated. Such primary human fibroblasts are readily obtained, for example, by punch
5 biopsy of the skin, or from tissues such as lung, liver or bone marrow. Fibroblasts useful in the invention also can be primary fibroblasts such as HFL-1 cells; the MRC-9 fibroblast cell line; and immortalized fibroblast cell lines including those immortalized with 4-nitroquinoline
10 1-oxide or ^{60}Co gamma rays such as the KMST-6, SUSM-1, and OUMS-24F lines (Iijima et al., Int. J. Cancer 66:698-702 (1996), which is incorporated herein by reference). Fibroblasts are particularly useful in the vaccines of the invention since fibroblasts are readily cultured and
15 propagated *in vitro* (Treco et al., "Fibroblast Cell Biology and Gene Therapy," in Chang (Ed.), Somatic Gene Therapy CRC Press, Boca Raton (1995), which is incorporated herein by reference).

A panel of vaccines produced from multiple
20 donor cells or cell lines can represent a variety of diseased cells and can express or have expressed a variety of different disease-associated antigens. For example, a panel of anti-tumor vaccines produced from multiple donor tumor cells, tumor cell lines or
25 transfected non-tumor cell lines can represent various histologic tumor types and express various known tumor antigens such as MZ2-E or mucin (see Finn, *supra*, 1993). Such a panel of anti-tumor vaccines, for example, can be maintained in a cell repository in a form readily
30 available for administration to an individual predisposed to developing a particular tumor type. The skilled artisan can select an appropriate genetically modified donor tumor cell from the panel based, for example, on the histologic type of tumor the individual has or is
35 predisposed to developing.

Bacterial cells also are useful in the cellular vaccines of the invention. Live bacterial vaccines using, for example, attenuated strains of bacteria are particularly useful since such live vaccines generally can confer a stronger, longer-lasting immune response than killed vaccines. Live bacterial vaccines can establish limited infections in the host that mimic the early stages of natural infection and lead to a natural immune response, and can confer extended immunity since the bacteria remain viable in the host for a long time. In addition, bacterial outer membrane proteins, lipopolysaccharides (LPS) and secreted bacterial toxins are strongly immunogenic and can act as natural adjuvants to enhance an immune response against a recombinant antigen. Furthermore, such live bacterial vaccines are easily administered, for example, orally (Francisco and Georgiou, *supra*, 1994).

A variety of avirulent bacterial strains have been developed for use as live vaccines. Bacteria useful in the cellular vaccines of the invention include *Salmonellae*, *Vibrio cholerae*, *Mycobacterium bovis*, *Streptococcus gordonii*, *Escherichia coli*, *shigella*, *lactobacillus*, *Listeria monocytogenes* and *Bacillus subtilis* (see, for example, Curtiss, "Attenuated *Salmonella* Strains as Live Vectors for the Expression of Foreign Antigens," in Woodrow and Levine (Ed.), New Generation Vaccines Marcel Dekker, Inc. (1990); Cardenas and Clements, Clin. Microbiol. Rev. 5:328-342 (1992); Cirillo et al., Clin. Infect. Dis. 20:1001-1009 (1995); and Fortaine et al., Res. Microbiol. 141:907-912 (1990), each of which is incorporated herein by reference). Bacteria useful in the vaccines of the invention also include *Shigella flexneri*, *Yersinia enterocolitica*, *bordetella pertussis* and *Staphylococcus xylosus* (Ryd et

al., Microbiol. Pathogen. 12:399-407 (1992); van Damme et al., Gastroenterol. 103:520-531 (1992); and Renauld-Mongenie et al., Proc. Natl. Acad. Sci., USA 93:7944-7949 (1996), each of which is incorporated herein
 5 by reference). Yeast cells such as *Saccharomyces cerevisiae* also can be useful in the vaccines of the invention, particularly in expressing membrane-bound fusion proteins that require post-translational modifications for activity.

10 *Salmonella* cells are particularly useful in the vaccines of the invention. *Salmonella* strains with mutations in genes such as *aroA*, *aroC*, *aroD*, *cya*, *crp*, *galE*, and *phoP/phoQ* are unable to sustain proliferation within mammalian cells. However, such live attenuated
 15 strains grow intracellularly long enough to stimulate an immune response. Attenuated *Salmonella* strains include nutritional auxotrophs such as those that are defective in biosynthesis of aromatic metabolites and that render the organism auxotrophic for PABA and
 20 2,3-dihydroxybenzoate. These attenuated strains have mutations in the *aro* genes, for example, deletions in one or more of the *aroA*, *aroC* or *aroD* genes. Deletions in adenylate cyclase (*cya*) and cyclic 3',5'-AMP receptor protein (*crp*) genes also are useful in generating
 25 attenuated *Salmonella* strains. Live attenuated *Salmonella* vaccines can be prepared using, for example, *S. typhimurium* strains such as Δ *aroA* Δ *aroD* BRD509, ISP1820 Δ *aroC* Δ *aroD*, Ty2 Δ *aroC* Δ *aroD* and Ty2 Δ *cya* Δ *crp* (see, for example, Tacket et al., Infect. Immun. 60:536-541
 30 (1992); Turner et al., Infect. Immun. 61:5374-5380 (1993); Dunstan et al., Infect. Immun. 64:2730-2736 (1996); Londoño et al., Vaccine 14:545-552 (1996), each of which are incorporated herein by reference).

Expression vectors for use in *Salmonella* include pKK233-2 and are well known in the art (Amann and Brosius, Gene 40:183-190 (1985); see, also, Anderson et al., "Development of Attenuated *Salmonella* Strains that

- 5 Express Heterologous Antigens" in Robinson et al., Methods in Molecular Medicine: Vaccine Protocols Humana Press, Inc. Totowa, NJ, each of which are incorporated herein by reference).

Listeria monocytogenes also are bacteria useful
 10 in the vaccines of the invention. *L. monocytogenes* based vaccines are useful, for example, to stimulate an immune response against influenza virus infection (Ikonomidis et al., Vaccine 15:433-440 (1997), which is incorporated herein by reference). Furthermore, *L. monocytogenes* can
 15 be engineered to express a disease-associated antigen or immunogenic epitope thereof, such as a tumor-associated antigen, for stimulation of an immune response to protect against or treat cancer (see, for example, Paterson and Ikonomidis, Curr. Opin. Immunol. 8:664-669 (1996), which
 20 is incorporated herein by reference).

An attenuated strain of *Mycobacterium bovis*, Bacillus Calmette-Guerin (BCG), also can be useful in the vaccines of the invention (Irvine and Restifo, Seminars in Cancer Biology 6:337-347 (1995); Stover et al, Nature
 25 351:456-460 (1991), each of which is incorporated herein by reference). BCG has been administered successfully as a tuberculosis vaccine, and components of the cell wall of BCG have powerful adjuvant activity. Mycobacterial expression vectors, which are useful for expressing a
 30 membrane-bound fusion protein and, if desired, a disease-associated antigen or immunogenic epitope thereof in a vaccine of the invention, are well known in art (Jacobs et al., Nature 327:532-535 (1987) and Snapper et al., Proc. Natl. Acad. Sci., USA 85:6987-6991 (1988),

each of which are incorporated herein by reference). One skilled in the art understands that these and other eukaryotic and prokaryotic host cells can be used in the vaccines and methods of the invention.

5 Expression vectors useful in the cellular vaccines of the invention include prokaryotic and eukaryotic expression vectors. Such expression vectors, including plasmids, cosmids, and viral vectors such as bacteriophage, baculovirus, retrovirus and DNA virus
10 vectors, are well known in the art (see, for example, Meth. Enzymol., Vol. 185, D.V. Goeddel, ed. (Academic Press, Inc., 1990) and Kaplitt and Loewy (Ed.), Viral Vectors: Gene Therapy and Neuroscience Applications (Academic Press, Inc., 1995), each of which are
15 incorporated herein by reference). Expression vectors contain the elements necessary to achieve constitutive or inducible transcription of a nucleic acid molecule encoding a membrane-bound fusion protein. Eukaryotic expression vectors that result in high levels of
20 sustained expression, such as vectors including cytomegalovirus (CMV), rous sarcoma virus (RSV), or simian virus 40 (SV40) promoter/enhancer elements, are particularly useful in the vaccines of the invention. Commercially available expression plasmids with strong
25 promoter/enhancer elements include pHOOK™-1, pHOOK™-2, pHOOK™-3, pcDNA3.1, pcDNA3.1/Hygro and pcDNA3.1/Zeo from Invitrogen (Carlsbad, CA). The pHOOK™-1, pHOOK™-2 and pHOOK™-3 expression plasmids include a nucleotide sequence encoding the human platelet derived growth
30 factor β receptor membrane attachment domain and, thus, are particularly useful in the vaccines of the invention (see Example I). One of ordinary skill in the art would

know which procaryotic or eukaryotic host systems are compatible with a particular vector.

An expression vector encoding a membrane-bound fusion protein can be introduced into a cell to produce a vaccine of the invention by any of a variety of methods known in the art and described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed, Vols 1 to 3, Cold Spring Harbor Laboratory Press, New York (1989), and in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (1994), each of which are incorporated herein by reference. Such methods include, for example, transfection via lipofection or electroporation to introduce recombinant expression vectors into eukaryotic cells. The introduction of the pHOOK™.1 expression vector into CT-26 cells is described in Example I.

In one embodiment, the cellular vaccine further includes a disease-associated antigen or immunogenic epitope thereof. Disease-associated antigens can be endogenous or exogenous to the cell and include tumor-associated antigens, autoimmune disease-associated antigens, infectious disease-associated antigens, viral antigens, parasitic antigens and bacterial antigens.

The term "disease-associated antigen," as used herein, means a molecule present on the surface of a diseased cell that can induce a cell-mediated or humoral immune response. Disease-associated antigens can be selectively expressed on particular disease cells, or can be expressed on both diseased and normal cells.

The term "immunogenic epitope thereof," as used herein in reference to a disease-associated antigen, means a portion of an antigen that functions as an antigenic determinant to induce a cell-mediated or

humoral immune response against the disease-associated antigen. Both T cell and B cell epitopes are encompassed within the term immunogenic epitope.

As used herein in reference to a
 5 disease-associated antigen and a cell, the term
 "endogenous" means a disease-associated antigen
 originating within the cell.

As used herein in reference to a
 10 disease-associated antigen and a cell, the term
 "exogenous" means a disease-associated antigen
 originating within the cell. Exogenous
 disease-associated antigens can be conveniently expressed
 in a cell having a membrane-bound fusion protein using
 recombinant methods well known in the art.

15 A variety of tumor-associated antigens are
 useful in the vaccines and methods of the invention (see
 Table 3). Such tumor-associated antigens include those
 which are tumor-specific as well as those which are
 tumor-selective. Tumor-associated antigens include p53
 20 and mutants thereof, Ras and mutants thereof, Bcr/Abl
 breakpoint peptides, HER-2/Neu, HPV E6, HPV E7,
 carcinoembryonic antigen, MUC-1, MAGE-1, MAGE-3, BAGE,
 GAGE-1, GAGE-2, N-acetylglucosaminyltransferase-V, p15,
 gp100, MART-1/MelanA, tyrosinase, TRP-1, β -catenin, MUM-1
 25 and CDK-4.

A tumor-associated antigen can be an oncogenic
 protein such as a nonmutated, overexpressed oncoprotein
 or a mutated, unique oncoprotein (Disis and Cheever,
Current Opin. Immunol. 8:637-642 (1996); Cornelis et al.,
 30 Curr. Opin. Immunol. 8:651-657 (1996, each of which are
 incorporated herein by reference). For example,
 mutations in p53 are present in about 50% of human
 malignancies, and a mutant p53 protein or peptide

fragment thereof can be a tumor-associated antigen useful in the invention (Yanuck et al., Cancer Res. 53:3257-3261 (1993); Noguchi et al., Proc. Natl. Acad. Sci., USA 92:2219-2223 (1995), each of which are incorporated
 5 herein by reference). A tumor-associated antigen useful in a vaccine of the invention also can be a normal p53 protein or peptide fragment thereof (Theobald et al., Proc. Natl. Acad. Sci., USA 92:11993-11997 (1995); Houbiers et al., Immunol. 23:2072-2077 (1993), each of
 10 which are incorporated herein by reference). Although p53 is present in both normal and tumor cells, vaccines including normal p53 peptides can promote a selective immune response against tumor cells due to the relative increased accumulation of p53 in the cytosol of tumor
 15 cells.

Mutations in Ras are present in about 15% of human malignancies. Mutant Ras proteins and peptides fragments thereof can be tumor-associated antigens useful in vaccines for treating such malignancies. Mutant Ras
 20 proteins usually have a single amino acid substitution at residue 12 or 61; Ras peptides spanning this mutant segment can be useful tumor-associated antigens (Cheever et al., Immunol. Rev. 145:33-59 (1995); Gjertsen et al., Lancet 346:1399-1400 (1995); Abrams et al., Seminars
 25 Oncol. 23:118-134 (1996); Abrams et al., Eur. J. Immunol. 26:435-443 (1996), each of which are incorporated herein by reference).

HER-2/neu also is a tumor-associated antigen, and peptides derived from the HER-2/neu proto-oncogene
 30 can be useful in the vaccines and methods of the invention (Disis et al., Cancer Res. 54:1071-1076 (1994); Bernhard et al., Cancer Res. 55:1099-1104 (1995); Mayordomo et al., Nature Med. 1:1297-1302 (1995), each of which is incorporated by reference herein). HER-2/neu is
 35 a growth factor receptor overexpressed in 30% of breast

and ovarian cancers and in a wide variety of other adenocarcinomas.

A tumor-associated antigen useful in the vaccines of the invention also can be the epidermal growth factor receptor (EGFR) or immunogenic epitope thereof, or a mutant EGFR variant or immunogenic epitope thereof. For example, the EGFR deletion mutant EGFRvIII is expressed in a subset of breast carcinomas and in non-small cell lung carcinomas and malignant gliomas. EGFRvIII disease-associated antigens, such as peptides corresponding to the novel EGFRvIII fusion junction, can be useful in stimulating an immune response against such tumors (Wikstrand et al., Cancer Res. 55:3140-3148 (1995); Moscatello et al., Cancer Res. 57:1419-1424 (1997), each of which are incorporated herein by reference). Thus, EGFR or EGFRvIII disease-associated antigens or immunogenic epitopes thereof can be useful in vaccines for the treatment of breast and lung carcinomas and malignant gliomas and to protect individuals at high risk from developing these cancers.

A tumor-associated antigen also can be a joining region segment of a chimeric oncoprotein such as Bcr-Abl (Ten-Bosch et al., Leukemia 9:1344-1348 (1995); Ten-Bosch et al., Blood 87:3587-3592 (1996), each of which are incorporated herein by reference).

A tumor-associated antigen useful in the vaccines of the invention also can be an E6 or E7 viral oncogene such as a human papilloma virus (HPV) E6 or E7 viral oncogene or immunogenic epitope thereof. For example, HPV16 is one of the major human papillomavirus types associated with cervical cancer, and immunogenic peptide epitopes encoded by HPV16 E6 and E7 can be useful in vaccines for the prevention and treatment of cervical carcinoma (see Ressing et al., J. Immunol. 154:5934-5943

(1995); Ressing et al., Cancer Res. 56:582-588 (1996), each of which are incorporated herein by reference).

A tumor-associated antigen useful in the vaccines of the invention also can be carcinoembryonic antigen (CEA). This antigen is highly expressed in the majority of colorectal, gastric and pancreatic carcinomas (Tsang et al., J. Natl. Cancer Inst. 87:982-990 (1995), which is incorporated herein by reference).

The MUC-1 mucin gene product, which is an integral membrane glycoprotein present on epithelial cells, also is a tumor-associated antigen useful in the invention. Mucin is expressed on almost all human epithelial cell adenocarcinomas, including breast, ovarian, pancreatic, lung, urinary bladder, prostate and endometrial carcinomas, presenting more than half of all human tumors (see, for example, Fin et al., Immunol. Rev. 145:61-89 (1995); Barratt-Boyes, Cancer Immunol. Immunother. 43:142-151 (1996), which are incorporated herein by reference). Vaccines of the invention containing full-length mucin or immunogenic epitopes thereof can therefore be used to protect against or treat epithelial cell adenocarcinomas such as breast carcinomas (Lalani et al., J. Biol. Chem. 266:15420-15426 (1991), which is incorporated herein by reference)

Minor histocompatibility antigens also can be used as tumor-associated antigens in the vaccines of the invention (Goulmy, Curr. Opin. Immunol. 8:75-81 (1996); Den Haan et al., Science 268:1478-1480 (1995); Wang et al., Science 269:1588-1590 (1995), each of which are incorporated herein by reference). For example, an HLA-A2 antigen can be used in the vaccines of the invention to treat human renal cell carcinomas (Brandle et al., J. Exp. Med. 183:2501-2508 (1996), which is incorporated herein by reference).

A variety of widely shared melanoma antigens also can be tumor-associated antigens useful in the vaccines of the invention (Robbins and Kawakami, Curr. Opin. Immunol. 8:628-636 (1996); Celli and Cole, Seminars Oncol. 23:754-758 (1996), each of which are incorporated herein by reference). For example, the MAGE-1, MAGE-2, MAGE-3, BAGE, GAGE-1 and GAGE-2 tumor-associated antigens or immunogenic epitopes thereof such as MZ2-E can be used in the vaccines of the invention for protection against and treatment of melanoma (van der Bruggen, Science 254:1643-1647 (1991), which is incorporated herein by reference). In normal adult tissue, the expression of MAGE related gene products is limited to testes and placenta; however, these tumor-associated antigens are expressed in a wide variety of tumor types, including breast carcinomas and sarcomas. A widely expressed melanoma tumor-associated antigen useful in the vaccines of the invention also can be, for example, N-acetylglucosaminyltransferase-V, which is expressed at significant levels in about 50% of melanomas and absent in normal tissues (Guilloux et al., J. Exp. Med. 183:1173-1183 (1996), which is incorporated herein by reference).

Melanoma tumor-associated antigens also can be differentiation antigens expressed by normal melanocytes. Such melanoma tumor-associated antigens include MART-1/MelanA; gp100; tyrosinase, the key enzyme in pigment synthesis; and the tyrosinase-related protein TRP-1 (gp75).

Unique melanoma antigens also can be tumor-associated antigens useful in the vaccines of the invention (Mumberg et al., Seminars in Immunol. 8:289-293 (1996), which is incorporated herein by reference). Such unique tumor-associated antigens include the MUM-1, β -catenin, and cyclin-dependent kinase CDK4 melanoma

Table 3		
EXEMPLARY DISEASE-ASSOCIATED ANTIGENS		
Antigen	Epitope	Reference
Non-melanoma antigens		
5 HER-2/ neu	IISAVVGIL(17) * KIFGSLAFL(18)	Peoples et al., <u>Proc. Natl. Acad. Sci. USA.</u> 92:432-436 (1995) Fisk et al., <u>J. Exp. Med.</u> 181:2109-2117 (1995)
HPV E6, HPV E7	YMLDLQPETT(19)	Ressing et al., <u>Cancer Res.</u> 56:582-588 (1996)
MUC-1	PDTRPAPGSTAPPAHGV TSA(20)	Fin et al., <u>Immunol Rev.</u> 145:61-89 (1995)
10	Tumor-specific, widely shared antigens	
MAGE-1	EADPTGHSY(21) SAYGEPRKL(22)	Traversari et al., <u>J. Exp. Med.</u> 176:1453-1457 (1992) Van der Bruggen et al., <u>Eur. J. Immunol.</u> 24:2134-2140 (1994)
MAGE-3	EVDPIGHL(23) FLWGPRALV(24)	Gaugler et al., <u>J. Exp. Med.</u> 179:91-21-930 (1994) Celis et al., <u>Proc. Natl. Acad. Sci. USA.</u> 91:2105-2109 (1994)
BAGE	AARAVFLAL(25)	Boel et al., <u>Immunity</u> 2:167-175 (1995)

Table 3

EXEMPLARY DISEASE-ASSOCIATED ANTIGENS

Antigen	Epitope	Reference
GAGE-1, GAGE-2	YRPRPRRY(26)	Van den Eynde et al., <u>J. Exp. Med.</u> 182:689-698 (1995)
GnT-V	VLDPVFIRC(27)	Guilloux et al., <u>J.</u> <u>Exp. Med.</u> 183:1173-1183 (1996)
p15	AYGLDFYIL(28)	Robbins et al., <u>J.</u> <u>Immunol</u> 154:5944-5950 (1995)
Melanocyte lineage proteins		
gp100	KTWGQYWQV(29) ITDQVPFSV(30) YLEPGPVTA(31) LLDGTATLRL(32) VLYRYGSFSV(33)	Kawakami et al., <u>J.</u> <u>Immunol</u> 154:3961-3968 (1995)
MART-1/ MelanA	AAGIGILTV(34) ILTVILGVL(35)	Kawakami et al., <u>J.</u> <u>Exp. Med.</u> 180:347-352 (1994) Castelli et al., <u>J.</u> <u>Exp. Med.</u> 181:363-368 (1995)
TRP-1 (gp75)	MSLQRQFLR(36)	Wang et al., <u>J. Exp.</u> <u>Med.</u> 183:1131-1140 (1996)

Table 3		
EXEMPLARY DISEASE-ASSOCIATED ANTIGENS		
Antigen	Epitope	Reference
5 Tyro- sinase	MLLAVLYCL(37)	Wölfel et al., <u>Eur J. Immunol</u> 24:759-764 (1994)
	YMNGTMSQV(38)	Wölfel et al., <i>supra</i> , (1994)
	SEIWRDIDF(39)	Brichard et al., <u>J. Immunol</u> 26:224-230 (1996)
	AFLPWHRLF(40)	Kang et al., <u>J. Immunol</u> 155:1343-1348 (1995)
	QNILLSNAPLGPQ(41) SYLQDSDPDSFQD(42)	Topalian et al., <u>J. Exp. Med.</u> 183:1965-1971 (1996)
Tumor-specific antigens		
β-catenin	SYLDSGIHF (43)	Robbins et al., <u>J. Exp. Med.</u> 183:1185-1192 (1996)
MUM-1	EEKLIVVLF (44)	Coulie et al., <u>Proc. Natl. Acad. Sci. USA.</u> 92:7976-7980 (1995)
10 CDK4	ACDPHSGHFV (45)	Wölfel et al., <u>Science</u> 269:1281-1284 (1995)
* SEQ ID NOS indicated in parenthesis		

antigens (Coulie et al., Proc. Natl. Acad. Sci., USA 92:7976-7980 (1995); Wolfel et al., Science 269:1281-1284 (1995); Robbins et al., J. Exp. Med. 183:1185-1192

(1996), each of which are incorporated herein by reference).

A disease-associated antigen of the invention can be a human immunodeficiency type I (HIV-1) antigen.

- 5 Such antigens include the gp120 envelope glycoprotein and immunogenic epitopes thereof such as the principal neutralization determinant (PND); gp160; and HIV-1 core protein derived immunogenic epitopes (see Ellis (Ed.), Vaccines: New Approaches to Immunological Problems
10 Stoneham, MA: Reed Publishing Inc. (1992), which is incorporated herein by reference).

- The vaccines of the invention also can contain autoimmune disease-associated antigens and can be useful in protecting against or treating diseases such as
15 rheumatoid arthritis, psoriasis, multiple sclerosis, systemic lupus erythematosus and Hashimoto's disease, type I diabetes mellitus, myasthenia gravis, Addison's disease, autoimmune gastritis, Graves' disease and vitiligo. Autoimmune disease-associated antigens can be,
20 for example, T cell receptor derived peptides such as V β 14, V β 3, V β 17, V β 13 and V β 6 derived peptides. Autoimmune disease-associated antigens also include annexins such as AX-1, AX-2, AX-3, AX-4, AX-4, AX-5 and AX-6, which are autoantigens associated with autoimmune
25 diseases such as systemic lupus erythematosus, rheumatoid arthritis and inflammatory bowel disease (Bastian, Cell. Mol. Life. Sci. 53:554-556 (1997), which is incorporated herein by reference). In addition, the annexins can be tumor-associated antigens useful in the vaccines of the
30 invention.

A variety of other disease-associated antigens also can be included in the vaccines of the invention. Such disease-associated antigens include viral, parasitic, yeast and bacterial antigens. For example,

Helicobacter pylori is the major causative agent of superficial gastritis and plays a central role in the etiology of peptic ulcer disease. Infection with *H. pylori* also appears increase the risk of gastric cancer.

- 5 The vaccines of the invention can be useful in protecting against *H. pylori* infection. Such vaccines can contain an *H. pylori* disease-associated antigen, for example, the urease protein, 90 kDa vacuolating cytotoxin (VacA), or 120 to 140 kDa immunodominant protein (CagA) of
10 *H. pylori*, or immunogenic epitopes thereof (Clyne and Drumm, Infect. Immun. 64:2817 (1996); Ricci et al., Infect. Immun. 64:2829-2833 (1996), each of which are incorporated herein by reference).

- The vaccines of the invention also can be used
15 to prevent the chronic inflammatory condition of tooth-supporting tissue that results in adult periodontal disease. In particular, *Porphyromonas gingivalis* is recognized as an important etiological agent of such disease, and disease-associated antigens derived from *P. gingivalis* can be included in the vaccines of the
20 invention for prevention and treatment of periodontal disease. *P. gingivalis* disease-associated antigens include the ArgI, ArgIA and ArgIB arginine-specific proteases of *P. gingivalis*, and immunogenic epitopes
25 thereof including the GVSPKVCKDVTVEGSNEFAPVQNL (SEQ ID NO:46) epitope (see, for example, Curtis et al., Infect. Immun. 64:2532-2539 (1996), which is incorporated herein by reference).

- Additional disease-associated antigens useful
30 in the vaccines of the invention include the MP65 antigen of *Candida albicans* (Gomez et al., Infect. Immun. 64:2577 (1996), which is incorporated herein by reference); helminth antigens; *Mycobacterial* antigens including

M. bovis and *M. tuberculosis* antigens; *Haemophilus* antigens; *Pertussis* antigens; cholera antigens; malaria antigens; influenza virus antigens; respiratory syncytial viral antigens; hepatitis B antigens; poliovirus
 5 antigens; herpes simplex virus antigens; rotavirus antigens and flavivirus antigens (Ellis, *supra*, 1992).

In a further embodiment, the vaccine contains a disease-associated antigen or immunogenic epitope
 10 thereof operatively fused to the membrane-bound fusion protein. Such vaccines are particularly useful in expressing an exogenous disease-associated antigen and have the advantage that only a single expression vector is utilized for expression of the membrane-bound fusion
 15 protein containing the non-antibody immunomodulatory molecule and for expression of the disease-associated antigen or immunogenic epitope.

Soluble cytokine-antigen fusion proteins have previously been expressed, such as those containing an
 20 idiotype antigen fused to GM-CSF, IL-2, IL-4, IFN- γ or an IL-1 β peptide (Tao and Levy, Nature 362:755-758 (1993); Hakim et al., *supra*, 1996; Chen et al., J. Immunol. 153:4775 (1994), which is incorporated herein by reference). Such soluble cytokine-antigen fusion
 25 proteins elicited an anti-idiotypic response that protected mice from tumor challenge and indicate that a variety of cytokines retain activity when expressed as fusion proteins.

In a vaccine of the invention, the
 30 membrane-bound fusion protein can contain an amino-terminal non-antibody immunomodulatory molecule operatively fused to a disease-associated antigen or immunogenic epitope thereof, which is operatively fused to a carboxyl-terminal heterologous membrane attachment

domain. Membrane-bound fusion proteins including a disease-associated antigen or immunogenic epitope thereof can be readily produced by recombinant methods. For example, the GM-CSF/PDGFR membrane attachment domain

5 construct described in Example I can be modified to include an operatively fused disease-associated antigen by cloning a nucleotide sequence encoding the antigen at the SalI site of pHOOKTM-1.GM-CSF.

A vaccine of the invention also can include a

10 second immunomodulatory molecule in membrane-bound or soluble form, in addition to the membrane-bound fusion protein that includes a non-antibody immunomodulatory molecule operatively fused to a heterologous membrane attachment domain. For example, combinations of

15 cytokines, which can produce an enhanced immune response such as a synergistic response as compared to the response produced by a single cytokine, are particularly useful in the vaccines of the invention. For example, in a vaccine of the invention, GM-CSF can be used in

20 combination with IL-4; IL-1 can be used in combination with TNF, IL-2, G-CSF, GM-CSF or IL-3; or IL-2 can be used in combination with IL-4. Similarly, IL-6 can be used in combination with, for example, IFN- γ , IL-4, IL-2 or M-CSF, and IL-7 can be used in combination with a

25 cytokine such as IL-2 or IL-4 (see Thomson, *supra*, 1994; Wakimoto et al., Cancer Vaccine 56:1828-1833 (1996), which is incorporated herein by reference).

A preferred vaccine of the invention includes GM-CSF in combination with IL-4. Such a cellular vaccine

30 of the invention can include, for example, IL-4 in membrane-bound or soluble form in addition to a membrane-bound fusion protein that contains GM-CSF operatively fused to a heterologous membrane attachment domain. Such a cellular vaccine of the invention also

35 can have GM-CSF in membrane-bound or soluble form in

addition to a membrane-bound fusion protein that contains IL-4 operatively fused to a heterologous membrane attachment domain.

In addition, a vaccine of the invention can contain, if desired, a B7-1 (CD80) or B7-2 (CD86) costimulatory molecule or a CD40 or CD40 ligand (Chen et al., Cell 71:1093-1102 (1992); Chen et al., J. Exp. Med. 179:523-532 (1994); Li et al., J. Immunol. 153:421-428 (1994); and Yang et al., J. Immunol. 154:2794-2800 (1995), each of which are incorporated herein by reference). A vaccine having a B7-1 or B7-2 costimulatory molecule in addition to a membrane-bound fusion protein including a non-antibody immunomodulatory molecule, such as GM-CSF, IL-2, IFN- γ or IFN- α , operatively fused to a heterologous membrane attachment domain.

The present invention also provides a method of modulating an immune response against a disease-associated antigen. In a method of the invention, an individual is administered a vaccine including a cell having a disease-associated antigen or immunogenic epitope thereof and a non-antibody immunomodulatory molecule operatively fused to a heterologous membrane attachment domain. The methods of the invention can be used alone, for example, to protect against or treat tumors, or can be used as adjuvant therapy following debulking of a tumor by conventional treatment such as surgery, radiotherapy and chemotherapy.

The methods of the invention for modulating an immune response can be used to treat a variety of diseases, conditions and disorders including tumors and cancers, autoimmune diseases, infectious diseases and disorders of bacterial, parasitic or viral etiology. In one embodiment, the methods of the invention can be used

to modulate an immune response for protection against or treatment of cancer, including cancers such as melanoma, colorectal cancer, prostate cancer, breast cancer, ovarian cancer, cervical cancer, endometrial cancer, glioblastoma, renal cancer, bladder cancer, gastric cancer, pancreatic cancer, neuroblastoma, lung cancer, leukemia and lymphoma. The methods of the invention also can be used to protect against or treat infectious diseases such as Acquired Immunodeficiency Syndrome (AIDS).

In addition, the methods of the invention can be used to protect against the development of or to treat existing autoimmune diseases such as rheumatoid arthritis, psoriasis, multiple sclerosis, systemic lupus erythematosus and Hashimoto's disease, type I diabetes mellitus, myasthenia gravis, Addison's disease, autoimmune gastritis, Graves' disease and vitiligo. Allergic reactions, such as hay fever, asthma, systemic anaphylaxis or contact dermatitis also can be treated using the methods of the invention for modulating an immune response.

A variety of diseases or conditions of bacterial, parasitic, yeast or viral etiology also can be prevented and treated using the methods of the invention for modulating an immune response. Such diseases and conditions include gastritis and peptic ulcer disease; periodontal disease; *Candida* infections; helminthic infections; tuberculosis; *Hemophilus*-mediated disease such as bacterial meningitis; *pertussis* virus-mediated diseases such as whooping cough; cholera; malaria; influenza infections; respiratory syncytial antigens; hepatitis; poliomyelitis; genital and non-genital herpes simplex virus infections; rotavirus-mediated conditions such as acute infantile gastroenteritis and diarrhea; and

flavivirus-mediated diseases such as yellow fever and encephalitis.

As disclosed herein, the methods of the invention can be used to treat an individual having one of such diseases or conditions or an individual suspected of having one of such diseases or conditions. The methods of the invention also can be used to protect an individual who is at risk for developing one of such diseases or conditions from the development of the actual disease. Individuals that are predisposed to developing particular diseases, such as particular types of cancer, can be identified using methods of genetic screening (see, for example, Mao et al., Canc. Res. 54(Suppl.):1939s-1940s (1994); Garber and Diller, Curr. Opin. Pediatr. 5:712-715 (1993), each of which is incorporated herein by reference). Such individuals can be predisposed to developing, for example, melanoma, retinoblastoma, breast cancer or colon cancer or disposed to developing multiple sclerosis or rheumatoid arthritis.

Immunomodulatory molecules useful in the methods of the invention include immunostimulatory and immunosuppressive molecules such as cytokines and heat shock proteins. A cytokine useful in the methods of the invention can be, for example, GM-CSF, G-CSF, IFN- γ , IFN- α , TNF- α , TNF- β , IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-12, lymphotactin or DC-CK1, another of the cytokines described hereinabove, or another cytokine molecule known in the art. Granulocyte macrophage colony stimulating factor (GM-CSF) is particularly useful in the methods of the invention.

Cells useful in the methods of the invention include prokaryotic and eukaryotic cells such as fibroblasts and tumor cells. As described above, a useful tumor cell can be, for example, a melanoma cell,

renal carcinoma cell, neuroblastoma cell, glioblastoma cell, lung cancer cell, colon cancer cell, breast cancer cell, prostate cancer cell, bladder carcinoma cell or plasmacytoma cell. In the methods of the invention, a
 5 cell can be autologous, allogeneic or xenogeneic to the individual to whom the vaccine is administered. For treatment of humans, allogeneic cells include HLA matched as well as unmatched cells. By HLA matched cells, it is meant that one or more of the major histocompatibility
 10 complex molecules on the vaccine cell is the same as one or more of the MHC molecules on cells the individual administered the vaccine cells. Such HLA matched allogeneic cells include, for example, HLA-A2 matched cells.

15 A variety of disease-associated antigens can be used to modulate an immune response against a disease-associated antigen. As discussed above, a disease-associated antigen can be endogenous or exogenous to the cell having the membrane-bound fusion protein.
 20 Such a disease-associated antigen can be, for example, a tumor-associated antigen, autoimmune disease-associated antigen, infectious disease-associated antigen, viral antigen, parasitic antigen or bacterial antigen. Tumor-associated antigens include p53 and mutants
 25 thereof, Ras and mutants thereof, a Bcr/Abl breakpoint peptide, HER-2/neu, HPV E6, HPV E7, carcinoembryonic antigen, MUC-1, MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, N-acetylglucosaminyltransferase-V, p15, gp100, MART-1/MelanA, tyrosinase, TRP-1, β -catenin, MUM-1 and
 30 CDK-4. Autoimmune disease-associated antigens include, for example, T cell receptor derived peptides. If desired, the disease-associated antigen or immunogenic epitope thereof can be operatively fused to the membrane-bound fusion protein.

The number of vaccine cells to be administered to an individual according to a method of the invention is the number of cells that can modulate an immune response against a disease-associated antigen. An effective number of vaccine cells to be administered can be determined using an assay for determining the activity of immunoeffector cells following administration of the vaccine to the individual or by monitoring the effectiveness of the therapy using well known *in vivo* diagnostic assays as described below. In general, a vaccine containing approximately 1×10^4 to 1×10^8 cells, and preferably 1×10^7 to 1×10^8 cells, is useful for modulating an immune response. One skilled in the art understands that the number of vaccine cells to be administered depends, for example, on the number of times the vaccine is to be administered and the level of response desired.

The vaccine cells of the invention can be administered with a pharmacologically acceptable solution such as physiological saline or with an appropriate adjuvant. Numerous pharmacologically acceptable solutions and adjuvants useful for immunization are known within the art. It is recognized that the vaccine cells of the invention should be stable in such solutions or adjuvants; for example, pharmacologically acceptable solutions which result in cell lysis are not useful in the methods of the invention.

Vaccine administration can be accomplished by any of various methods including subcutaneous, intradermal or intramuscular injection, injection directly into tumor lesions, and oral administration. One skilled in the art understands that oral administration is particularly useful for prokaryotic vaccines such as *Salmonella* vaccines. Intradermal or subcutaneous administration, or a combination thereof, is

particularly useful for administration of a vaccine containing membrane-bound GM-CSF. For treatment of tumors, administration can be at the site of a tumor or can be at a location other than the primary tumor site.

5 Multiple routes of administration, as well as administration at multiple sites to increase the area contacted by the vaccine, also are envisioned by the present invention. It is recognized that booster vaccines administered, for example, every several months,
10 also can be useful in modulating an immune response against a disease-associated antigen according to a method of the invention.

One skilled in the art would know that the effectiveness of therapy can be determined by monitoring
15 immune functions in the patient. In anti-tumor therapy, for example, the cytolytic activity of immune effector cells against a patient's cancer cells can be assayed using the methods described in Example II. In addition, the size or growth rate of a tumor can be monitored *in*
20 *vivo* using methods of diagnostic imaging. By monitoring the patient during therapy, the physician would know whether to use repeated administration of a vaccine of the invention.

Further provided herein is a nucleic acid
25 molecule including a nucleotide sequence encoding a non-antibody immunomodulatory molecule operatively linked to a heterologous nucleotide sequence encoding a membrane attachment domain functional at neutral or basic pH. The non-antibody immunomodulatory molecule can be an
30 immunostimulatory or immunosuppressive molecule. Cytokines and heat shock proteins, for example, are immunomodulatory molecules useful in the nucleic acid molecules of the invention. Such a cytokine can be, for example, GM-CSF, G-CSF, IFN- γ , IFN- α , TNF- α , TNF- β , IL-1.
35 IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-12, lymphotactin

and DC-CK1. The cytokine GM-CSF is particularly useful in the nucleic acid molecules of the invention.

A nucleic acid molecule including a nucleotide sequence encoding an non-antibody immunomodulatory molecule operatively linked to a heterologous nucleotide sequence encoding a membrane attachment domain functional at neutral or basic pH can further include, if desired, an operatively linked nucleotide sequence encoding a disease-associated antigen or immunogenic epitope thereof.

Nucleic acids encoding cytokine-diphtheria toxin fusion proteins have been previously described (vanderSpek et al, Mol. Cell. Biochem. 138:151-156 (1994); Murphy and vanderSpek, Seminars Cancer Biol. 6: 259-267 (1995), each of which are incorporated herein by reference). These fusion proteins contain a hydrophobic domain of diphtheria toxin, which function at acidic pH in delivery from endocytic vesicles to the cytosol. However, such diphtheria toxin hydrophobic domains do not function at neutral pH, for example, in membrane binding to a plasma membrane. Thus, nucleic acids encoding diphtheria toxin fusion proteins are excluded from the nucleic acids of the invention.

The disease-associated antigen can be, for example, a tumor-associated antigen, autoimmune disease-associated antigen, infectious disease associated antigen, viral antigen, parasitic antigen or bacterial antigen. Tumor-associated antigens include p53 and mutants thereof, Ras and mutants thereof, Bcr/Abl breakpoint peptides, HER-2/neu, HPV E6, HPV E7, carcinoembryonic antigen, MUC-1, MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, N-acetylglucosaminyltransferase-V, p15, gp100, MART-1/MelanA, tyrosinase, TRP-1, β -catenin, MUM-1 and CDK-4 as well as other tumor-associated antigens

known in the art. Autoimmune disease-associated antigens include, for example, T cell receptor derived peptides.

The invention further provides a nucleic acid molecule including a nucleotide sequence encoding a non-antibody immunomodulatory molecule operatively linked to a heterologous nucleotide sequence encoding a membrane attachment domain, provided that the membrane attachment domain is not derived from diphtheria toxin.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Production of cellular vaccines containing membrane-bound GM-CSF

This example describes the preparation and use of a cellular vaccine including a membrane-bound GM-CSF fusion protein.

Preparation of the pHOOK™-1.GM-CSF expression construct

In order to isolate the mouse GM-CSF cDNA, total RNA was prepared from Concanavalin A-stimulated spleen cells isolated from Balb/c mice (Jackson Labs, Bar Harbor, ME). Stimulated spleen cells were lysed in Trizol™ (Gibco-BRL; Gaithersburg, MD) at a concentration of 5×10^6 cells/ml Trizol™ and frozen at -70°C . Cells were then thawed, 200 μl chloroform added and the sample vortexed 15-30 seconds. The sample was then incubated at room temperature for 10 minutes before centrifuging at $12,000 \times g$ for 15-20 minutes at 4°C . The colorless aqueous phase was removed to a new tube, and 5-20 μl of glycogen added from a stock solution of 20 $\mu\text{g}/\mu\text{l}$. After adding 500 μl isopropyl alcohol, the sample was incubated

for 1 hour at 15-30°C and then centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was removed, and the pellet washed with 1 ml of 75% ethanol by vortexing and subsequently centrifuging at 7,5000 x g for 5 minutes at 4°C. The washed pellet was then air dried for 10 minutes and dissolved in 30 µl water.

cDNA synthesis was performed using SuperScript™ II (Gibco-BRL) essentially as follows. Briefly, oligo dT primers (0.5 µg) were added to 2.5 µg RNA sample. The volume was adjusted to 11 µl with water, and the samples incubated at -70°C for 10 minutes, before placing on ice for at least 1 minute. The following mixture was prepared and added to the sample: 2 µl 10X PCR buffer from SuperScript™ II kit; 2 µl 25 mM MgCl₂; 1 µl 10 mM dNTP; and 2 µl 0.1 M DTT. The mixture was incubated at 42°C for 5 minutes before the addition of 1 µl (200 units) SuperScript™ II reverse transcriptase and a further incubation at 42°C for 50 minutes. The reaction was stopped by incubation for 15 minutes at -70°C, followed by chilling on ice. After centrifuging briefly, the mixture was incubated with RNaseH for 20 minutes at 37°C, and the volume adjusted to 100 µl with water.

Murine GM-CSF was amplified essentially as follows. The 5' PCR murine GM-CSF primer SEQ ID NO:47 contains an ApaI restriction site and has the sequence 5'-GCGGAGGGGCCCTAGCACCCACCCGCTCACCCATCACT-3'. The 3' PCR murine GM-CSF primer SEQ ID NO:8 contains a SalI restriction site and has the sequence 5'-ACCGCGGTCGACTTTTTTGGACTGGTTTTTGCATTCAAAGGGG-3'. These GM-CSF specific primers were used in a reaction containing 5 µl of 10 µM stocks of each GM-CSF primer; 2 µl of GM-CSF cDNA isolated from Concanavalin A-stimulated mouse spleen cells; 4 µl 10 mM cDNA (Gibco-BRL); 10 µl 10X Taq polymerase buffer (Perkin Elmer); 10 µl 25 mM

MgCl₂; and 69 μ l water. The sample was heated to 100°C for 5 minutes, cooled to 80°C and incubated for 5 minutes before adding 2 units of Taq polymerase. The sample was then amplified in a Perkin Elmer Cetus DNA thermocycler for 35 cycles with an annealing temperature of 55°C.

As shown in Figure 1, the pHOOKTM-1 vector (Invitrogen, San Diego, CA) contains the coding sequence for a single-chain antibody located between the murine kappa chain signal peptide and a platelet-derived growth factor receptor (PDGFR) membrane attachment domain coding sequence. The pHOOKTM-1 vector also contains sequences coding for ampicillin, kanamycin and neomycin resistance. The murine GM-CSF PCR fragment was purified and cloned into the ApaI and SalI sites of pHOOKTM-1 to produce pHOOKTM-1.GM-CSF. JM109 cells were transformed with the ligation mixture, and restriction digest analysis subsequently used to identify clones that were positive for the GM-CSF insert. A large-scale preparation of endotoxin-free pHOOKTM-1.GM-CSF plasmid material was prepared using the Qiagen plasmid purification system (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. A control construct for expression of soluble GM-CSF was prepared similarly but contains a stop codon prior to the SalI site.

25 Transfections and clone selection

Mouse colon adenocarcinoma CT-26 cells were obtained from the Sidney Kimmel Cancer Center (SKCC, La Jolla, CA) and are described in Fakhrai et al., Human Gene Therapy 6:591-601 (1995) and Shawler et al., J. Immunol. Emphasis Tumor Immunol. 17:201-208 (1995), each of which are incorporated herein by reference. The CT-26 cells were transfected by electroporation using Superfect (Qiagen) according to the manufacturer's instructions. Clones were selected with 1 mg/ml G418 (Gibco-BRL) in

RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, L-glutamic acid and β -mercaptoethanol. Transfected cell lines were maintained in selective media.

5 Reverse transcriptase polymerase chain reaction

Cells expressing membrane-bound GM-CSF by FACS were further analyzed for the presence of GM-CSF mRNA by RT-PCR as described above. RNA was prepared with Trizol™ (Gibco-BRL), and cDNA was isolated as described in the product protocol. Primers SEQ ID NOS:47 and 48, described above, amplify a fragment of 370 bp from the pHOOK™-1.GM-CSF template. As shown in Figure 3, of twelve G418-resistant CT-26 transfectants, eight had the 370 bp PCR amplified fragment, indicating that these lines were positive for GM-CSF mRNA. The 370 bp fragment indicative of GM-CSF mRNA is evident in the positive control Concanavalin A stimulated Balb/c spleen cells, but absent from wild type CT-26 cells as expected.

Flow cytometry

After 4 weeks of selection in G418 containing media, colonies positive for GM-CSF mRNA were screened for the expression of membrane-bound GM-CSF by flow cytometry on a Becton Dickinson FACSTAR (Becton Dickinson, San Jose, CA). Briefly, cells were harvested and washed in 1% FBS in phosphate-buffered saline (PBS) and incubated with anti-GM-CSF fluorescein-labeled monoclonal antibody (Pharmingen, La Jolla, CA) for 1 hour on ice. The cells were washed with 1% FBS in PBS and analyzed for expression of membrane-bound GM-CSF. Of eight G418 resistant lines having murine GM-CSF mRNA by RT-PCR analysis, six were shown by fluorescence activated

cell sorting (FACS) to have cell surface expressed GM-CSF.

Double Stain FACS analysis of pHOOK™-1.GM-CSF transfected CT-26 cells

5 The staining of transfected cells with antibodies specific for GM-CSF was performed essentially as described above, with the addition of a simultaneous staining antibody control for various markers (anti-H-2K^d class I MHC marker, anti-IFN- γ , anti-I-A^d class II MHC marker).

10 The results of several of the transfected clones demonstrated a positive staining with the anti-GM-CSF antibody, while antibodies against other marker proteins were negative. The positive control anti-H-2K^d antibody showed a positive signal on all pHOOK™-1.GM-CSF transfected CT-26 cells at the same magnitude as the wild-type controls. The negative control anti-IFN- γ and anti-I-A^d antibodies were significantly lower or negative on all cells.

20 Representative FACS analysis of the pHOOK™-1.GM-CSF CT-26 transfectant "C3" is shown in Figure 4. Staining with anti-H-2K^d antibody, shown along the X-axis of panels B, C, and D, yielded a positive signal as expected for this positive control antibody.

25 Staining with anti-IL-4 is shown along the Y axis of panel B, and staining with anti-IFN- γ is shown along the Y axis of panel C. As shown in these panels, the pHOOK™-1.GM-CSF transfected CT-26 cells did not express cell-surface IL-4 or IFN- γ . However, as shown in

30 panel D, staining with anti-GM-CSF was positive. These results demonstrate that GM-CSF is expressed on the cell surface of pHOOK™-1.GM-CSF transfected cells.

Radioimmunoprecipitation assay

Surface expression of GM-CSF is further assayed by surface iodination and immunoprecipitation of pHOOK™-1.GM-CSF transfected cells essentially as described in Kranz et al., Proc. Natl. Acad. Sci., USA 81:573-577 (1984), which is incorporated herein by reference. Briefly, transfected cells are surface iodinated with Iodo-Beads® (Pierce Chemicals, Rockford, IL) according to the manufacturer's instructions. After anti-GM-CSF antibody (Pharmingen) is conjugated to Affi-gel® (BioRad Laboratories, Richmond, CA), the conjugated Affi-gel® beads are incubated with the iodinated cells for one hour on ice. Subsequently, the mixture is incubated with Triton X-100 to a final concentration of 0.1% for a further 15 minutes on ice. To remove unbound proteins, the Affi-gel® beads are washed five times with ice cold 1% FBS in PBS. SDS loading dye is added to the washed beads, and the mixture heated to 100°C for 2 minutes. The immunoprecipitated products are analyzed by electrophoresis on a 15% SDS-PAGE gel, which is dried and analyzed by autoradiography.

CT-26 transfectants expressing membrane-bound GM-CSF/PDGFR fusion protein have a labeled protein of about 20 kDa. This protein is absent from the negative control, wild type CT-26 cells.

Bone marrow proliferation assay

In order to test membrane-bound GM-CSF for biological activity, pHOOK™-1.GM-CSF transfected CT-26 cells are assayed for the ability to stimulate proliferation of fresh bone marrow cells as described in Bulkwill (Ed), Cytokines: A Practical Approach Oxford

Press (1995) pp 247-268, which is incorporated herein by reference. Briefly, pHOOK™-1.GM-CSF transfected CT-26 cells or control non-transfected CT-26 cells (2×10^5 cells per well) are incubated with an equal number of syngeneic mouse bone marrow cells in 96-well plates. After two days, wells are pulsed with 1 μ Ci of 3 H-thymidine and incubated for another day. The cells are harvested onto filters using a vacuum manifold, and the amount of 3 H-thymidine subsequently analyzed. Bone marrow proliferation, as indicated by the amount of 3 H-thymidine, is significantly greater for cells incubated with pHOOK™-1.GM-CSF transfected CT-26 cells as compared to non-transfected CT-26 cells.

EXAMPLE II

Tumor protection using cellular vaccines containing membrane-bound GM-CSF

This example demonstrates that a cellular vaccine expressing a membrane-bound GM-CSF/PDGFR fusion protein can be used for tumor protection.

Tumor protection experiments

pHOOK™-1.GM-CSF transfected CT-26 cells are assayed for the ability to protect mice inoculated with wild type CT-26 colon adenocarcinoma cells essentially as described in Shawler et al., J. Immunol. Emphasis Tumor Immunol. 17:201-208 (1995), which is incorporated herein by reference. Briefly, pHOOK™-1.GM-CSF transfected CT-26 cells are irradiated with 25,000 rads using a JLShepard and Associates Model 109-85 Irradiator with a 60 Cobalt source. Balb/c mice (Jackson Labs) are vaccinated with 1×10^4 irradiated pHOOK™-1.GM-CSF transfected CT-26 cells and boosted twice weekly with 1×10^4 cells.

Subsequently, mice are challenged with 1×10^4 live, wild type CT-26 cells in the opposite flank. Tumor dimensions are scored every other day to evaluate the efficacy of the vaccine.

- 5 The pHOOK™-1.GM-CSF transfected CT-26 vaccine cells significantly reduce tumor growth as compared to animals vaccinated with wild-type CT-26 cells and as compared to unvaccinated animals. In addition, the pHOOK™-1.GM-CSF transfected CT-26 vaccine cells
- 10 significantly reduce tumor growth as compared to animals vaccinated with CT-26 cells producing a soluble form of murine GM-CSF.

Cytotoxic T lymphocyte assays

- Spleens are removed from mice vaccinated and
- 15 boosted with irradiated pHOOK™-1.GM-CSF transfected CT-26 cells. In order to detect the presence of cytotoxic T lymphocytes specific for CT-26 tumor cells, a single cell suspension is made from the spleens and used as the effector cells in a standard 4 hour chromium release
- 20 assay (Kranz et al., Proc. Natl. Acad. Sci., USA 81:7922-7926 (1984), which is incorporated herein by reference). Briefly, the target cells are wild type CT-26 cells that are passively labeled with $125 \mu\text{Ci } ^{51}\text{Cr}$ in complete medium for 1 hour in a 37°C water bath. The
- 25 CT-26 target cells are washed 3 times in complete media and incubated with increasing numbers of spleen cells from vaccinated animals for 4 hours in a 5% CO_2 humidified incubation chamber. The percent specific lysis (chromium release) is calculated as
- 30 $100\% \times (\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{spontaneous}}) / (\text{cpm}_{\text{maximal}} - \text{cpm}_{\text{spontaneous}}).$

T cell proliferation assays

Vaccinated animals are assayed for an increased population of T cells that are specific for wild-type CT-26 cells. Essentially, spleens of vaccinated animals are removed, and a single cell suspension prepared as described above. Approximately 2×10^5 spleen cells are incubated with increasing numbers of irradiated, wild-type CT-26 cells for 3 days in 5% CO₂ at 37°C. On day 2, the cells are pulsed with 1 μ Ci of ³H-thymidine. The cells are harvested onto glass fiber filters, and the amount of ³H-thymidine counted. The amount of stimulation (stimulation index) is calculated as the amount of ³H-thymidine of the experimental wells divided by the amount of ³H-thymidine of T cells exposed only to complete media.

ELISAs and FACS analysis of serum antibodies

Vaccinated animals are assayed for the ability to elicit specific antibodies to CT-26. Vaccinated animals are bled retro-orbitally to obtain serum. The serum is diluted 1:50, 1:100 and 1:500 and incubated with whole wild-type CT-26 cells. The cells are subsequently washed 2 times with cold PBS containing 1% BSA and incubated with a FITC-conjugated secondary antibody specific for mouse immunoglobulin. The cells are washed once more and analyzed by FACS for staining.

For ELISA assays, CT-26 cells are fixed with glutaraldehyde in a 96-well plate format and washed with PBS containing 1% BSA. The fixed cell monolayer is subsequently blocked with PBS containing 1% BSA for 1 hour at room temperature. The cells are stained with diluted antisera from vaccinated animals for 1 hour at room temperature. After washing with blocking buffer,

HRP-conjugated goat anti-mouse Ig secondary antibody is added for 1 hour at room temperature. The secondary reagent is washed, and the fixed cells reacted with HRP substrate (Kirkegaard and Perry Labs, Bethesda, MD).

- 5 All journal article, reference, and patent citations provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference.

- 10 Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention. It should be understood that various modifications can be made without departing from the
15 spirit of the invention. Accordingly, the invention is limited only by the following claims.

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What is claimed is:

1. A vaccine, comprising a cell having a membrane-bound fusion protein comprising a non-antibody immunomodulatory molecule operatively fused to a heterologous membrane attachment domain.

2. The vaccine of claim 1, wherein said non-antibody immunomodulatory molecule is an immunostimulatory molecule.

3. The vaccine of claim 1, wherein said non-antibody immunomodulatory molecule is an immunosuppressive molecule.

4. The vaccine of claim 1, wherein said non-antibody immunomodulatory molecule is selected from the group consisting of cytokine and heat shock protein.

5. The vaccine of claim 4, wherein said cytokine is selected from the group consisting of:

granulocyte macrophage colony stimulating factor (GM-CSF),

granulocyte colony stimulating factor (G-CSF),

interferon γ (IFN- γ),

interferon α (IFN- α),

tumor necrosis factor- α (TNF- α),

tumor necrosis factor- β (TNF- β),

interleukin-1 (IL-1),

interleukin-2 (IL-2),

interleukin-3 (IL-3),

interleukin-4 (IL-4),

interleukin-6 (IL-6),

interleukin-7 (IL-7),

interleukin-10 (IL-10),

interleukin-12 (IL-12),

lymphotactin and

dendritic cell chemokine 1 (DC-CK1).

6. The vaccine of claim 5, wherein said cytokine is GM-CSF.

7. The vaccine of claim 1, wherein said cell
5 is a prokaryotic cell.

8. The vaccine of claim 1, wherein said cell
is a eukaryotic cell.

9. The vaccine of claim 8, wherein said
eukaryotic cell is a fibroblast

10 10. The vaccine of claim 8, wherein said
eukaryotic cell is a tumor cell.

11. The vaccine of claim 10, wherein said
tumor cell is selected from the group consisting of
melanoma cell, renal carcinoma cell, neuroblastoma cell,
15 glioblastoma cell, lung cancer cell, colon tumor cell,
breast tumor cell, prostate tumor cell, bladder carcinoma
cell and plasmacytoma cell.

12. The vaccine of claim 1, wherein said cell
further has a disease-associated antigen or immunogenic
20 epitope thereof.

13. The vaccine of claim 12, wherein said
disease-associated antigen is endogenous to said cell.

14. The vaccine of claim 12, wherein said
disease-associated antigen is exogenous to said cell.

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15. The vaccine of claim 12, wherein said disease-associated antigen is selected from the group consisting of tumor-associated antigen, autoimmune disease-associated antigen, infectious disease-associated antigen, viral antigen, parasitic antigen and bacterial antigen.

16. The vaccine of claim 15, wherein said tumor-associated antigen is selected from the group consisting of p53 and mutants thereof, Ras and mutants thereof, a Bcr/Abl breakpoint peptide, HER-2/neu, HPV E6, HPV E7, carcinoembryonic antigen, MUC-1, MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, N-acetylglucosaminyltransferase-V, p15, gp100, MART-1/MelanA, tyrosinase, TRP-1, β -catenin, MUM-1 and CDK-4.

17. The vaccine of claim 15, wherein said autoimmune disease-associated antigen is a T cell receptor derived peptide.

18. The vaccine of claim 12, wherein said disease-associated antigen or immunogenic epitope thereof is operatively fused to said membrane-bound fusion protein.

19. A method of modulating an immune response against a disease-associated antigen, comprising administering to an individual a vaccine comprising a cell having:

- (a) a disease-associated antigen or immunogenic epitope thereof and
- (b) a non-antibody immunomodulatory molecule operatively fused to a heterologous membrane attachment domain.

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26. The method of claim 19, wherein said cell is a eukaryotic cell.

27. The method of claim 26, wherein said eukaryotic cell is a fibroblast.

28. The method of claim 26, wherein said eukaryotic cell is a tumor cell.

29. The method of claim 28, wherein said tumor cell is selected from the group consisting of melanoma cell, renal carcinoma cell, neuroblastoma cell, glioblastoma cell, lung cancer cell, colon cancer cell, breast cancer cell, prostate cancer cell, bladder carcinoma cell and plasmacytoma cell.

30. The method of claim 19, wherein said disease-associated antigen is endogenous to said cell.

31. The method of claim 19, wherein said disease-associated antigen is exogenous to said cell.

32. The method of claim 19, wherein said disease-associated antigen is selected from the group consisting of a tumor-associated antigen, autoimmune disease-associated antigen, infectious disease-associated antigen, viral antigen, parasitic antigen and bacterial antigen.

33. The method of claim 32, wherein said tumor-associated antigen is selected from the group consisting of p53 and mutants thereof, Ras and mutants thereof, a Bcr/Abl breakpoint peptide, HER-2/neu, HPV E6, HPV E7, carcinoembryonic antigen, MUC-1, MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, N-acetylglucosaminyltransferase-V, p15, gp100, MART-1/MelanA, tyrosinase, TRP-1, β -catenin, MUM-1 and CDK-4.

34. The method of claim 32, wherein said autoimmune disease-associated antigen is a T cell receptor derived peptide.

35. The method of claim 19, wherein said disease-associated antigen or immunogenic epitope thereof is operatively fused to said membrane-bound fusion protein.

5 36. A nucleic acid molecule, comprising a nucleotide sequence encoding an non-antibody immunomodulatory molecule operatively linked to a heterologous nucleotide sequence encoding a membrane attachment domain functional at neutral or basic pH.

10 37. The nucleic acid molecule of claim 36, wherein said non-antibody immunomodulatory molecule is an immunostimulatory molecule.

15 38. The nucleic acid molecule of claim 36, wherein said non-antibody immunomodulatory molecule is an immunosuppressive molecule.

 39. The nucleic acid molecule of claim 36, wherein said non-antibody immunomodulatory molecule is selected from the group consisting of cytokine and heat shock protein.

20 40. The nucleic acid molecule of claim 39, wherein said cytokine is selected from the group consisting of GM-CSF, G-CSF, IFN- γ , IFN- α , TNF- α , TNF- β , IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-12, lymphotactin and DC-CK1.

25 41. The nucleic acid molecule of claim 40, wherein said cytokine is GM-CSF.

30 42. The nucleic acid molecule of claim 36, further comprising an operatively linked nucleotide sequence encoding a disease-associated antigen or immunogenic epitope thereof.

43. The nucleic acid molecule of claim 42,
wherein said disease-associated antigen is selected from
the group consisting of tumor-associated antigen,
autoimmune disease-associated antigen, infectious disease
5 associated antigen, viral antigen, parasitic antigen and
bacterial antigen.

44. The nucleic acid molecule of claim 43,
wherein said tumor-associated antigen is selected from
the group consisting of p53 and mutants thereof, Ras and
10 mutants thereof, Bcr/Abl breakpoint peptides, HER-2/neu,
HPV E6, HPV E7, carcinoembryonic antigen, MUC-1, MAGE-1,
MAGE-3, BAGE, GAGE-1, GAGE-2,
N-acetylglucosaminyltransferase-V, p15, gp100,
MART-1/MelanA, tyrosinase, TRP-1, β -catenin, MUM-1 and
15 CDK-4.

45. The nucleic acid molecule of claim 43,
wherein said autoimmune disease-associated antigen is a
T cell receptor derived peptide.

46. A nucleic acid molecule, comprising a
20 nucleotide sequence encoding a non-antibody
immunomodulatory molecule operatively linked to a
heterologous nucleotide sequence encoding a membrane
attachment domain, provided that said membrane attachment
domain is not derived from diphtheria toxin.

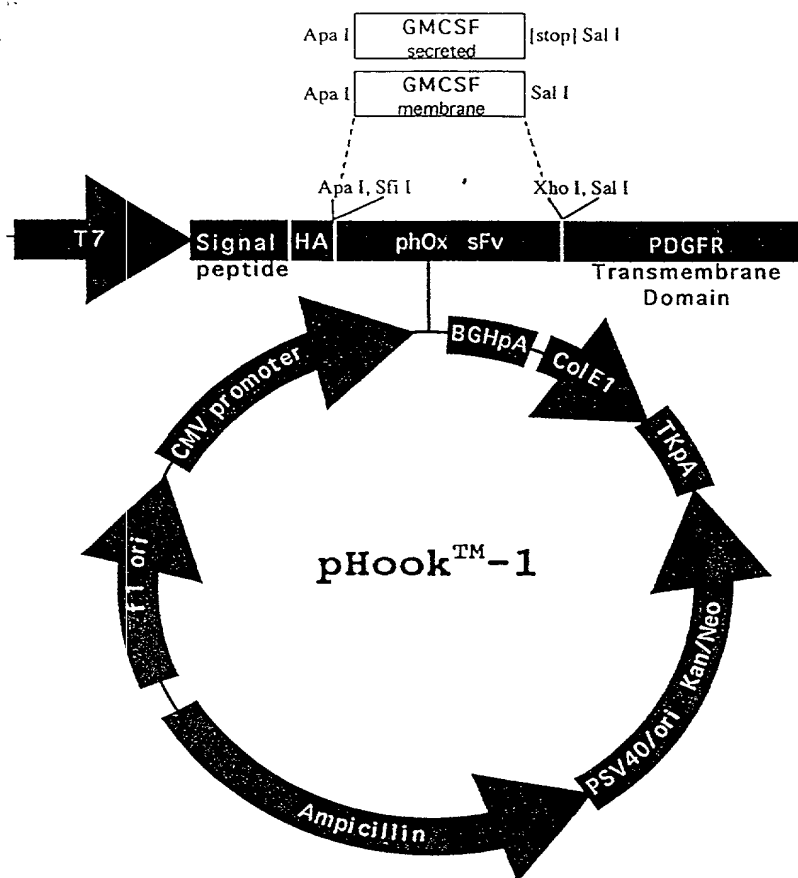
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MEMBRANE-BOUND CYTOKINE COMPOSITIONS AND
METHODS OF MODULATING AN IMMUNE RESPONSE USING SAME

ABSTRACT

5 The present invention provides a cellular
vaccine having a membrane-bound fusion protein that
includes a non-antibody immunomodulatory molecule
operatively fused to a heterologous membrane attachment
domain. Non-antibody immunomodulatory molecules useful
in the invention include immunostimulatory and
10 immunosuppressive molecules such as cytokines. In one
embodiment, the invention provides a cellular vaccine
having a membrane-bound fusion protein that includes a
non-antibody immunomodulatory molecule operatively fused
to a heterologous membrane attachment domain and,
15 additionally, a disease-associated antigen or immunogenic
epitope thereof. Further provided by the invention are
methods of modulating an immune response against a
disease-associated antigen by administering to an
individual a cellular vaccine having a membrane-bound
20 fusion protein that includes a non-antibody
immunomodulatory molecule operatively fused to a
heterologous membrane attachment domain.

A.



B.

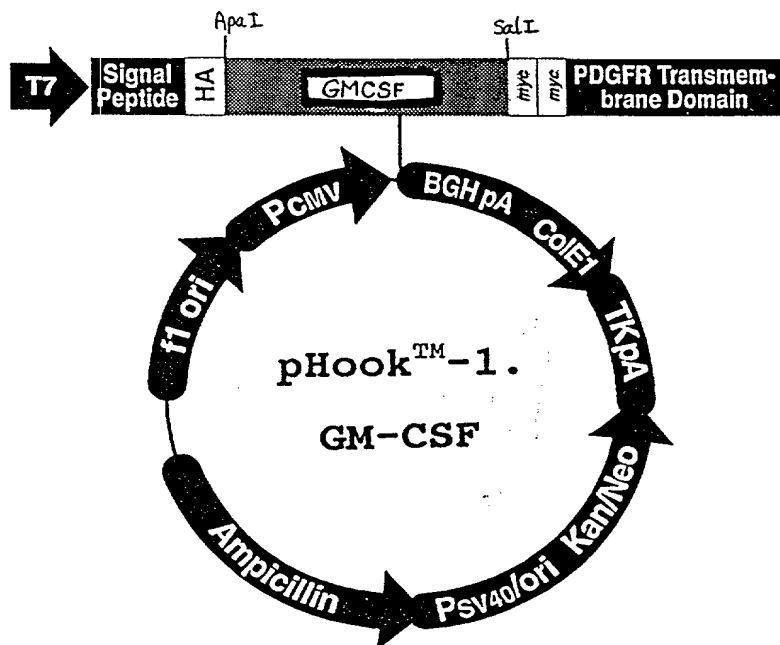


Figure 1

10 20 30 40 50 60 70 80 90 100
 ATGAGACAG ACACACTCT GCTATGGGTA CTGCTGCTT GGGTTCACAG TTCCACTGCT GACTATCCAT ATGATGTTCC AGATTATGCT GGGGCCACAG
 M E T D T L L L W V L L L W V P G S T G D Y P Y D V P D Y A G A Q
 ← *murine kappa light chain signal sequence* →
 110 120 130 140 150 160 170 180 190 200
 * * * * *
 CACCACCCG CTCACCCAT ACTGTCAACC GGCCTTGGA GCATGTAGAG GCCATCAAG AACCCCTGAA CCTCCGTGAT GACATGCCCTG TCACGTTGAA
 A P T R S P I T V T R P W K H V E A I K E A L N L L D D M P V T L N
 ← *mouse GmC5f* →
 210 220 230 240 250 260 270 280 290 300
 * * * * *
 TGAGAGGTA GAATGCTCT CTACAGATT CTCTTCACAG AAGTACACAT GTGTGCAGAC CCGCTGAG ATATTGAGC AGGTCTACG GGGCAATTTC
 E E V E V V S N E F S F K K L T C V Q T R L K I F E Q G L R G N F
 310 320 330 340 350 360 370 380 390 400
 * * * * *
 ACCAACTCA AGGCGCCTT GAACATGACA GCCAGTACT ACCAGACATA CTGCCCCCA ACTCCGGAAG CGGACTGTGA AACACAGTT ACCACTATG
 T K L K G A L N M T A S Y Y Q T Y C P P T P E T D C E T Q V T T Y
 410 420 430 440 450 460 470 480 490 500
 * * * * *
 CGGATTTCAT AGACACCTT AAAACCTTTC TGACTGATAT CCCCTTGAA TGCAAAAC CAGGCCAAGA AGTCAGACGA CAAAATCA TTCTGAGA
 A D F I D S L K T F L T D I P F E C K K P G Q K
 ← *mouse GmC5f* →
 510 520 530 540 550 560 570 580 590 600
 * * * * *
 GGATCTGAAT GCTGTGGGC AGACACGCA GGAAGTCATC GTGTGCGCAC ACTCCTTGCC CTTAAGTGT GTGTGATCT CAGCAATCCT GGCCCTGTG
 D L N A V G Q D T Q E V I V V P H S L P F K V V V I S A I L A L V
 ← *PDGF β transmembrane domain* →
 610 620 630 640 650 660 670 680 690
 * * * * *
 GTGCTACCA TCATCTCCCT TATCATCTTC ATCATGCTTT GGCAGAGAA GCCACGTTAG GCGGCCGCTC GAGATCACC TGACATGTGC CTTCATG
 V L T I I S L I I L I M L W Q K K P R * A A A R D Q P R L C L L
 ← *transmembrane domain* →

[illegible]

Inventor: William Soo Hoo
Docket #: P-IM 2442



Figure 3

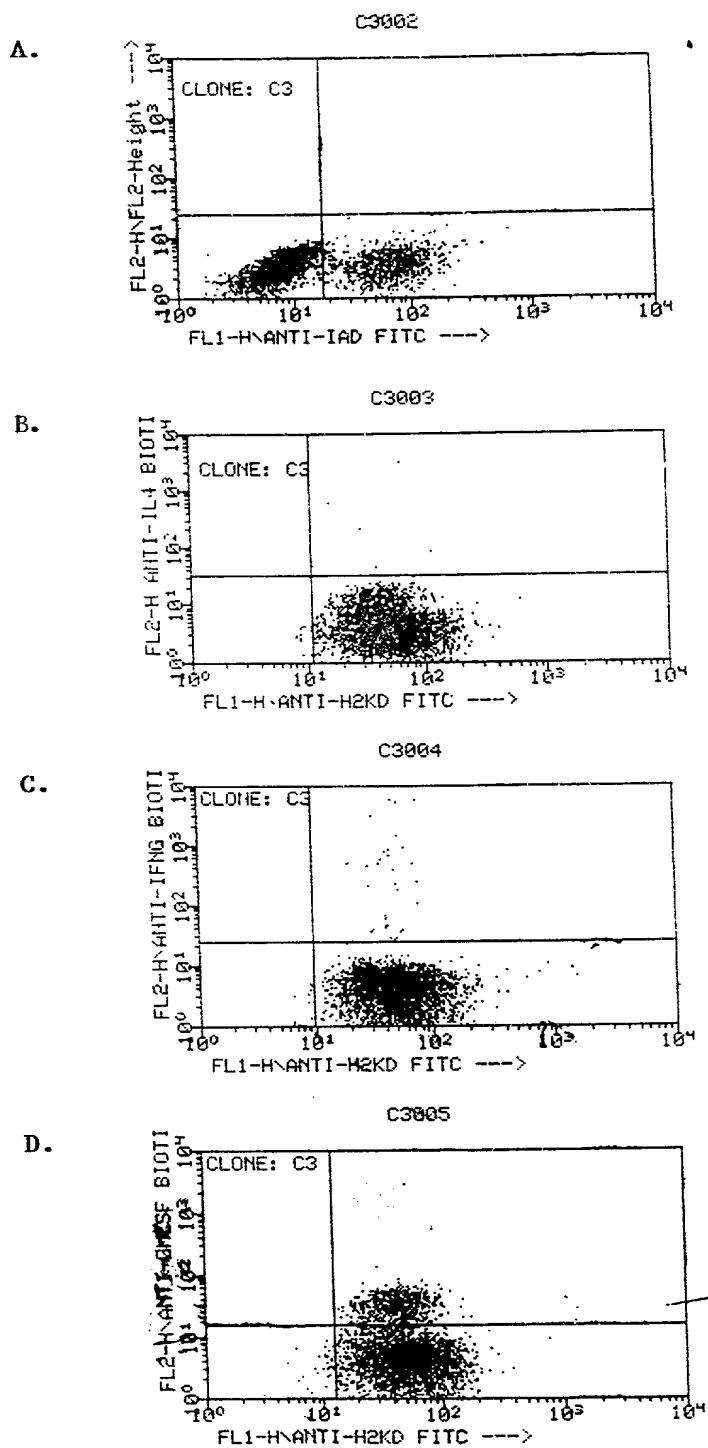


Figure 4